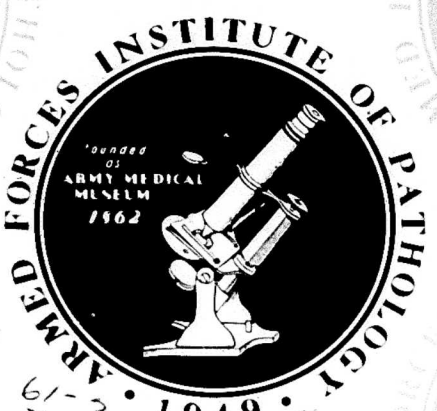
# AD 259 234










# AFIP

## ANNUAL PROGRESS REPORTS ON RESEARCH FOR ARMY MEDICAL R&D CONTRACTS

1 July 1960 - 30 June 1961

ARMED FORCES INSTITUTE OF PATHOLOGY

Washington 25, D. C.

# ANNUAL PROGRESS REPORT

REPORTS CONTROL SYMBOL MEDDH - 288

1 July 1960 -- 30 June 1961

Qualified requestors may obtain copies of this report from the Armed Services Technical Information Agency (ASTIA).

## TABLE OF CONTENTS

## DISTRIBUTION LIST

Four copies to:

US Army Medical Research & Development Command, Department of the Army, Washington 25, D.C.

Ten copies to:

Armed Services Technical Information Agency, Arlington Hall Station, Arlington 12, Virginia

One copy to:

US AMEDS Combat Development Group, Forest Glen Section, WRAMC, Washington 12, D.C.
Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington 12, D. C.
Medical Equipment Development Laboratory, Fort Totten, Flushing 59, L. I., New York
US Army Prosthetics Research Laboratory, Walter Reed Army Medical Center, Washington 12, D. C.
US Army Medical Research Unit, Kuala Lumpur, Malaya
US Army Medical Unit, Fort Detrick, Maryland
US Army Tropical Research Medical Laboratory, APO 851, New York, N. Y.
US Army Medical Research Laboratory, Fort Knox, Kentucky
US Army Surgical Research Unit, Brooke Army Medical Center, Fort Sam Houston, Texas
US Army Medical Research & Nutrition Laboratory, Fitzsimons General Hospital, Denver 30, Colorado
US Army Medical Research Unit, Europe, APO 180, New York, N.Y.
US Army Medical Research Unit, Box 2011, Balboa Heights, Canal Zone
Valley Forge General Hospital, Phoenixville, Pennsylvania (ATTN: Librarian)
Letterman General Hospital, Presidio of San Francisco, Californis (ATTN: Librarian)
William Beaumont General Hospital, El Paso, Texas (ATTN:Librari
Brooke General Hospital, Brooke Army Medical Center, Fort Sam Houston, Texas (ATTN: Librarian)
Fitzsimons General Hospital, Denver, Colorado (ATTN: Librarian)
Madigan General Hospital, Tacoma, Washington (ATTN: Librarian)
Walter Reed General Hospital, Walter Reed Army Medical Center, Washington 12, D. C. (ATTN: Librarian)
The Historical Unit, U.S.Army Medical Service, Forest Glen Section, Walter Reed Army Medical Center, Washington 12, D.C.
United States Army, Europe, APO 403, New York, New York (ATTN: Surgeon)
United States Army, Alaska, APO 949, Seattle, Washington (ATTN: Surgeon)

DISTRIBUTION LIST (continued)

United States Army, Pacific, APO 958, San Francisco, California
(ATTN: Surgeon)
United States Army, Caribbean, Fort Amador, Canal Zone
(ATTN: Surgeon)

ANNUAL PROGRESS REPORT

Title Page

Project No. (6x60-11-001) Nutrition: Radiation and Sterilization of Food

Name and Address of Reporting Installation:

Armed Forces Institute of Pathology
Washington, D. C.

Name of Department and Division:

Department of Pathology, Veterinary Division

Period Covered by the Report: 1 July 1960 - 30 June 1961

Professional Authors of the Report:

Principal Investigator: Col. F. D. Maurer, VC

Assistant: Lt. Col. Martin A. Ross, VC

Reports Control Symbol: (RCS-MEDDH-288)

Security Classification:

(Unclassified)

## ABSTRACT

Project No. 6x60-11-001 Title: Nutrition: Radiation and Sterilization of Food

Name and Address of Reporting Installation: Armed Forces Institute of Pathology Washington, D. C.

Period Covered by the Report: 1 July 1960 - 30 June 1961

Authors: Lt. Col. M. A. Ross, VC; Col. F. D. Maurer, VC

Reports Control Symbol: (RCS MEDDH-288)

Security Classification: Unclassified

## SUMMARY

This report concerns the activity of the Armed Forces Institute of Pathology on the research project promulgated by the Research and Development Command, Office of the Army Surgeon General and designed to produce the experimental data relating to the possible toxicity of food sterilized by irradiation. Contractors have fed the irradiated foods to rats, dogs or monkeys for a period of two years. At the time of death, sacrifice or completion of the feeding period, tissues are prepared for histologic examination by the contractor's pathologist. Paraffin blocks, duplicate slides and protocols are forwarded to the AFIP for processing in accord with our responsibilities. About 85% of the total material anticipated has been received at the Armed Forces Institute of Pathology. This includes material from 2662 rats, 215 dogs and 19 monkeys.

Preliminary inspection of the data from five dog feeding programs reveal no evidence that ingestion of the food items tested produced histopathologic changes. The rat and monkey feeding programs are still too incomplete to permit summary statements concerning possible effects.

## BODY OF REPORT

**Project No. 6x60-11-001 Title: Nutrition: Radiation & Sterilization of Food.**

**Description: This is a continuation of the program developed by the R & D Division of the Office of the Army Surgeon General designed to produce experimental data relating to the possible toxicity of foods sterilized by irradiatio Seventeen contractors are feeding irradiated foods to rats, dogs, or monkeys for two-year periods. Animals that die during the course of the study or that are sacrificed at the termination of the feeding period are autopsied. Tissue are taken from each animal, processed and examined histologically.. The paraf blocks, a duplicate set of slides and a complete protocol, including the histopathological findings for each animal, are forwarded to the Armed Forces Institute of Pathology for file, review and IBM coding.**

About 85% of the total material anticipated has been received at the Armed Forces Institute of Pathology. This includes material from 2662 rats, 215 dogs and 19 monkeys.

| | RATS | | DOGS | |
|---|---|---|---|---|
| Contractors | No. Recd. | % of total anticipated | No. Recd. | % of total anticipate |
| Alabama | 142 | 100% | 24 | 100% |
| Cornell | | | 56 | 66% |
| Georgia | | | 24 | 100% |
| Hazelton | 93 | 100% | | |
| M.I.T. | | | 0 | 0 |
| Med. College of Va. | | | 54 | 100% |
| Oregon | 651 | 68% | | |
| Syracuse | 644 | 100% | | |
| Texas A & M | 305 | 100% | | |
| Univ. Ill. | | | 24 | 100% |

| Contractors | RATS No. Recd. | RATS % of total anticipated | DOGS No. Recd. | DOGS % of total anticipated |
|---|---|---|---|---|
| Univ. Calif. | 328 | 100% | | |
| Univ. Miami | 209 | 69% | 0 | 0% |
| Univ. Mich. | 158 | 100% | | |
| Vanderbilt | 101 | 59% | 10 | 28% |
| VPI | | | 23 | 100% |
| Wisconsin | 105 | 100% | | |
| | Monkeys | | | |
| Vanderbilt | 19 | 53% | | |

All protocols except for 175 have been initially coded. IBM processing is proceeding in good order, including the analysis of data for completed contracts.

The completed preliminary report for the feeding study conducted by the Georgia Coastal Plain Experiment Station was submitted with the semi-annual progress report for the period 15 September 1960 - 15 March 1961. The following preliminary reports are scheduled for early completion and are expected to be submitted with the semi-annual progress report for the period 15 March 1961 - 15 September 1961.

| Contractor | Test Food(s) | Species of Animals |
|---|---|---|
| Univ. Mich. | Potatoes | Rats |
| Texas A & M | Green beans and chicken | Rats |
| Med. Coll. Va. | Green beans and Fruit compote | Dogs |
| VPI | Shrimp and carrots | Dogs |
| Alabama | Codfish and sweet potatoes | Dogs |
| Univ. Ill. | Flour and Beef | Dogs |

There has been no evidence noted by observation that the feeding of foods preserved by irradiation causes untoward tissue changes. This observation is particularly true for the dog feeding studies as the numbers of animals are sufficiently small to permit individual review. The lesions noted in material from rat feeding programs have been similar in all respects to those reported in previous annual reports.

Significantly, the lesions noted previously as being predominant in the Sprague-Dawley strain, i.e. arteritis, chronic nephrosis, and glomerulonephritis, have been attributed to the aging process by Simms and Berg.[1] The sinusoidal ectasia of adrenals particularly in the Wistar strains has been similar to that illustrated by Jayne.[2]

It is expected that all outstanding material generated by this vast program, with few exceptions, will be received at the Armed Forces Institute of Pathology during the coming fiscal year. The preliminary reports on each contract will be prepared as rapidly as possible and submitted with the semi-annual progress reports due on 15 March and 15 September respectively. It is hoped the final summation report can be initiated and the project basically completed during fiscal 1963.

Summary and Conclusions:

About 85% of the total material expected on this program has been received. This is a total of 2896 accessioned cases, all but 175 of which has been initially reviewed and coded.

General impressions gained from the material reviewed to date offer no suggestion that the consumption of irradiated foods produces histologic change nor intensifies naturally occurring disease or aging lesions.

The completion of many individual contract reports is anticipated during

fiscal 1962 and initiation of the final summary reports during fiscal 1963.

List of Publications:

1. Semi-Annual Progress Report for period 15 September 1960 - 15 March 1961
2. Report of Findings, Georgia Coastal Plains Experiment Station.

References:

1. Henry S. Simms, Ph.D. and Benjamin N. Berg, M.D.: Longevity and the onset of lesions in male rats. Journal of Gerontology, vol 12, Section A, No. 3, July 1957, 244-252.

2. Edgar P. Jayne, Ph.D.: Histochemical and Degenerative Changes in the Adrenal Cortex of the Rat with Age. Journal of Gerontology, vol. 12, Section 14, No. 1, Jan. 1957, 2-7.

# ANNUAL PROGRESS REPORT

## Title Page

PROJECT NO. 6X61-09-001 - Studies on Immunization

TASK NO. 2 Labeled Antibody Technique for Electron Microscopy

NAME & ADDRESS OF REPORTING INSTALLATION:

Armed Forces Institute of Pathology

Washington 25, D. C.

NAME OF DEPARTMENT AND DIVISION:

Department of Pathology

Division of Basic Sciences

Immunochemistry Branch

PERIOD COVERED BY THE REPORT: 1 July 1960 - 30 June 1961

PROFESSIONAL AUTHORS OF THE REPORT:

Felix Borek, Ph.D.*

Richard E. Hartman, Ph.D.**

Roberta S. Hartman, Ph.D.**

*Assigned as principal investigator on 13 September 1960, for the period of the temporary absence of Dr. A.M.Silverstein

**Walter Reed Army Institute of Research

REPORTS CONTROL SYMBOL: (RCS-MEDDH-288)

SECURITY CLASSIFICATION: Unclassified

## ABSTRACT

PROJECT NO. 6X61-09-001 TITLE: Studies on Immunization

TASK NO. 2 TITLE: Labeled Antibody Technique for Electron Microscopy

NAME AND ADDRESS OF REPORTING INSTALLATION:

Armed Forces Institute of Pathology

Washington 25, D. C.

PERIOD COVERED BY THE REPORT: 1 July 1960 - 30 June 1961

AUTHORS: Felix Borek, Ph.D., Richard E. Hartman, Ph.D., and Roberta S. Hartman, Ph.D.

REPORTS CONTROL SYMBOL: (RCS - MEDDH - 288)

SECURITY CLASSIFICATION: Unclassified

### SUMMARY

The development of methods for the preparation and characterization of ferritin-antibody globulin conjugates is being continued. A new, improved conjugation method and its application to viral antibodies is described. Previously developed purification methods are being applied to various conjugates, and the search for new methods continues. The application of the conjugates to the labeling of viruses, studied by electron microscopy, is described.

Studies on Immunization
Labeled Antibody Technique
Annual Progress Report
30 June 1961

## INTRODUCTION

The work on The Development and Application of a Labeled Antibody Technic for Electron Microscopy was begun in 1958 and, after promising preliminary results were obtained, it was submitted for support on 19 February 1959, and approved on 24 April 1959. The aim of investigations involved is the development of a method for the labeling of antibody molecules with the electron-opaque protein ferritin. Antibody labeled in this way can be employed as a specific immunohistochemical stain for antigenic substances on the subcellular level with the use of the electron microscope.

## PROGRESS

As already outlined in the progress report for the period 1 July 1959 - 30 June 1960, the project may be divided into three phases: 1) the development of methods for coupling ferritin to antibody; 2) the characterization and purification of ferritin-antibody conjugates; 3) the application of ferritin-antibody conjugates to the staining and localization of antigens on the electron-microscopic level.

The progress of the work in these three areas is outlined below:

1. Coupling Procedures. The coupling of ferritin to antibody which would result in a conjugate applicable to the specific staining of the homologous antigen, requires a bifunctional chemical compound which would combine with both components of the conjugate and thus form a stable bridge between them. Until recently, the best reagent for this purpose was considered to be m-xylylene diisocyanate, originally introduced by Singer (Nature, 183, 1523, 1959). The use of this reagent, however, leads to a partial loss by precipitation of the proteins involved, and to a decrease in antibody activity. Last year Singer reported (unpublished) that the use of toluene 1, 4-diisocyanate results in an improved coupling. We confirmed this finding, but continued at the same time our search for more satisfactory coupling methods.

It is well known that the mildest chemical reaction of proteins resulting in minimum denaturation and in minimum loss of antibody activity is diazonium coupling. Our earlier attempts to use bis-diazotized benzidine for the ferritin-antibody coupling were not successful, presumably because the reaction of both functional groups with ferritin was too rapid to provide a sufficient number of ferritin-antibody linkages. More recently we turned to bis-diazotized dianisidine, a dimethoxy derivative

of benzidine. The o-methoxy groups are known to slow down the diazonium coupling reaction (Hammett, "Physical Organic Chemistry," N. Y., 1940, p. 314). Moreover, it has been reported by Pearse (Histochemistry," London, 1954, p. 255) that at acid pH only one end of bis-diazotized dianisidine undergoes coupling, whereas at higher pH levels both ends of the molecule are able to react. The use of this reagent, accompanied by changing the pH of the reaction from 5 to 9, proved to lead to a successful coupling of ferritin with anti-tobacco mosaic virus antibody globulin. No protein was lost by precipitation, and the conjugate could be easily separated from unreacted globulin and ferritin. It is our intention to adapt this new coupling procedure to other ferritin-antibody systems.

2. Characterization and Fractionation of Conjugates. The experiments on application of some of our ferritin-antibody conjugates to electron-microscopic identification of plant viruses, performed by our collaborators, Drs. Richard and Roberts Hartman, Biophysics Section, Department of Molecular Biology, WRAIR, showed that the separation of the conjugates from uncoupled ferritin and globulin is essential for the elimination of the nonspecific "background" staining. We were able to achieve such a separation by means of a continuous-flow paper electrophoresis at pH 8.6. This method proved to be efficient and reliable. The ferrocyanide method for iron determination was adapted to assay the electrophoretic fractions for

ferritin content and thus to determine the efficiency of coupling. The antibody titers of the purified ferritin-antiprotein conjugates were determined by the tanned-cell hemagglutination method.

Following immune globulins have been coupled to ferritin, with subsequent purification of the conjugates: rabbit anti-ovalbumin, anti-bovine gamma globulin, anti-Rh factor, anti-southern bean mosaic virus and anti-tobacco mosaic virus. In one instance, a conjugate of anti-TMV with ferritin was prepared from antibody specifically purified by precipitating the virus and dissociating the virus-antibody complex. All the conjugates have been characterized by the use of moving-boundary electrophoresis, immunoelectrophoresis, ultracentrifuge and the Ouchterlony agar-diffusion method.

We are currently investigating the possibility of using other methods, not involving a specialized apparatus, for the separation of the conjugates from uncoupled globulin and ferritin. Application of column chromatography with DEAE-cellulose adsorbent (originally introduced for serum fractionation by Drs. Sober and Peterson of the National Cancer Institute, Bethesda, Maryland) was tried for this purpose. By using buffers (pH 6.4 and 7.1) of increasing ionic strength as eluents, we could easily separate the uncoupled globulin from the conjugate and the uncoupled ferritin, but the latter two components were always eluted together. According to Dr. Peterson, whom we consulted about this problem, this might be due to the fact that ferritin has a much larger molecular surface area than globulin

and thus it confers on the conjugate adsorptive properties very similar to those of uncoupled ferritin. We intend to try the application of other chromatographic materials for this purpose, particularly those which are selective with respect to the molecular weight of proteins rather than those adsorptive properties.

3. Application of Conjugates to the Identification of Antigenic Sites in the Electron Microscope. During the past year considerable effort has gone into attempts to develop specimen preparative technics using ferritin-globulin conjugates which are satisfactory for the identification of antigenic structures in the electron microscope. To date our studies have been confined to attempts to demonstrate specific attachment of ferritin to a variety of small viruses, using highly purified viral preparations. In many instances electron micrographs have been obtained in which the ferritin was found preponderantly associated with the recognizable viral body when the virus had been treated with its homologous ferritin-globulin conjugate. However, when attempts were made to produce satisfactory external controls, i. e. similar preparations made under conditions such that ferritin should not be found associated with the viral bodies, the results were inconsistent.

As a result of these experiments it was obvious that considerable refinement in the procedure will be required. In order to provide a system which contains an internal control, a mixture of southern bean mosaic virus (SBMV) and tobacco mosaic virus (TMV) was used. When such a mixture is treated with anti SBMV ferritin-globulin the

spherical SBMV particles should be labeled and the TMV rods should not. The converse should occur when anti-TMV ferritin-globulin is used. Using a specimen preparative technique in which the viral ferritin-antibody mixture was presumably washed free of nonspecific ferritin by dialyzing against saline using a 10 mμ millipore filture as the membrane, electron micrographs were obtained in which the SBMV was specifically labeled when the mixture was treated with anti-SBMV ferritin-globulin conjugate. However, when anti-TMV ferritin-globulin conjugate was used both viruses were labeled. As these ferritin-globulin conjugates precipitated only the homologous virus, the failure of the reverse system to show an internal control is believed to be due to the inadequacy of those steps in the technique designed to remove material held by weaker forces than those of specific adsorption, this inadequacy being important in the case of the low-titer anti-TMV. More effective means for removal of nonreactive conjugate are being sought.

4. Plans for Future Work. More efficient methods of conjugation of ferritin to globulin will be studied, primarily with the aim of increasing the relative antibody content which is essential for improving the quality of labeled specimens used for the electron microscopic work. Attemps will be also made to develop more straight-foward methods of the purification of the conjugates.

Further work will aim at developing preparative methods (fixation, washing, filtration, etc.) which would provide unequivocal controls. This will require an effective removal of all but trace amounts of unreacted conjugates without affecting the integrity of the antigen-antibody systems to be observed.

## SUMMARY

The development of methods for the preparation and characterization of ferritin-antibody globulin conjugates is being continued. A new, improved conjugation method and its application to viral antibodies is described. Previously developed purification methods are being applied to various conjugates, and the search for new methods continues. The application of the conjugates to the labeling of viruses, studied by electron microscopy, is described.

List of Publications:

F. Borek and A. M. Silverstein, Characterization and Purification of Ferritin-Antibody Conjugates, J. Immunol., in press.

F. Borek, A New Two-Stage Method for Cross-Linking Proteins, in preparation.

Distribution:

As per OTSG Admin. Ltr. 705-2

# ANNUAL PROGRESS REPORT

## Title Page

PROJECT NO. 6X60-01-001 - Internal Medicine

TASK NO. 3 A Study of Motor End Plates in Animals and Man

NAME & ADDRESS OF REPORTING INSTALLATION:

Armed Forces Institute of Pathology

Washington 25, D. C.

NAME OF DEPARTMENT:

Office of the Army Deputy Director

PERIOD COVERED BY THE REPORT: 1 July 1960 - 30 June 1961

PROFESSIONAL AUTHORS OF THE REPORT:

Principal Investigator: Joe M. Blumberg, Colonel, MC, USA

Assistants: Walter C. Bauer, Captain, MC, USA

Sumner I. Zacks, M.D.

REPORTS CONTROL SYMBOL: RCS-MEDDH-288

SECURITY CLASSIFICATION: Unclassified

# ABSTRACT

PROJECT NO. 6X60-01-001 TITLE: Internal Medicine

TASK NO. 3 TITLE: A Study of Motor End Plates in Animals and Man

NAME AND ADDRESS OF REPORTING INSTALLATION:

Armed Forces Institute of Pathology

Washington 25, D. C.

PERIOD COVERED BY THE REPORT: 1 July 1960 - 30 June 1961

AUTHORS: Joe M. Blumberg, Colonel, MC, USA

Walter C. Bauer, Captain, MC, USA

Sumner I. Zacks, M. D.

REPORTS CONTROL SYMBOL: RCS-MEDDH-288

SECURITY CLASSIFICATION: Unclassified

## SUMMARY

A study of the fine structure of motor end plates from five patients with myasthenia gravis has shown definite anatomical abnormalities in both the neural and muscular components of the synapse. These changes, characterized by distorted and deranged secondary synaptic clefts, focal absence of muscle cell surface membrane, decreased numbers of sole plate mitochondria and loss of fine structure detail in the terminal axon branch, are inter-

preted as degenerative changes in the motor end plate and have not been seen in normal human synapses. The number of affected end plates and the severity of the degenerative changes roughly parallels the clinical state of the disease in these five cases.

Studies with ferritin-labeled Staph-aureus antibodies demonstrated satisfactory electron microscopic localization of the antibody within a capsule-like material of the organism. In a similar manner ferritin-labeled Botulinus neurotoxin B was found localized to the "basement membrane" material in the primary and secondary synaptic clefts of mouse motor end plates.

Observations on the fine structure changes in the motor end plates and skeletal muscle of mice following denervation have been made for periods up to 24 weeks post-denervation. Degeneration and retraction of the terminal axon branch is complete within one week, however, the sole plate sarcoplasm and secondary synaptic folds remain intact for at least five weeks. Examination of the muscle cell shows a regular spectrum of fine structure changes during the post-denervation period.

Observations on the histogenesis of chick motor end plates have begun.

BODY OF REPORT

PROJECT NO. 6X60-01-001 - Internal Medicine

TASK NO. 3 A Study of Motor End Plates in Animals and Man

DESCRIPTION:

1. Electron Microscopic Observations on Neuromuscular Junctions and Skeletal Muscle in Myasthenia Gravis.

a. End plate changes. Following the successful development of techniques for fixation of skeletal muscle biopsies for electron microscopy and the demonstration of the fine structure of the normal human motor end plate[1], biopsies from patients with myasthenia gravis were examined to determine if any changes in the fine structure could be found. Extensive physiologic and pharmacologic data have accumulated indicating that the neuromuscular junction is the major site of the defect in patients with this disease[2].

To the present time six biopsies from myasthenic patients have been obtained: five cases through the courtesy of Dr. Kermit Osserman of Mt. Sinai Hospital, New York, and one case from the Johns Hopkins Hospital through the cooperation of Doctors McGehee Harvey and Richard Johns. The former five biopsy specimens have been studied extensively and come from patients with the following brief histories:

Case 1 - A 2½ year old male child with progressive generalized weakness, characteristic Jolly reaction, positive Tensilon test twice while under sedation and improvement on Prostigmin and Mestinon therapy.

Case 2 - A 42-year old woman with slowly progressive myasthenia for 21 years who was hospitalized for regulation of therapy (Mestinon).

Case 3 - A 30-year old woman with severe and rapidly progressive weakness of recent onset. A Prostigmin test was positive. The patient underwent a thymectomy because of the rapid progression of the disease.

Case 4 - A 41-year old Negro woman with myasthenia of 2 years duration who had recent attacks of severe weakness requiring hospitalization. The patient underwent

surgery for an anterior mediastinal mass and a thymoma was removed. Three weeks following discharge the patient was readmitted in another myasthenic crisis and died within a few hours.

Case 5 - A 72-year old woman with an 8-year history of myasthenia beginning with severe bulbar weakness. On Prostigmin and Mestinon therapy her weakness was reasonably well controlled until 6 months ago when increasing weakness in the extremities, dysphagia and dyspnea could not be controlled by increasing the medication.

Formalin-fixed, paraffin embedded material was available on all five cases and showed no discernable abnormalities.

Biopsies from the vastus internus (Cases 1 and 2), intercostal muscle (Cases 3 and 4) and palmaris longus (Case 5) were obtained for electron microscopy without recourse to methylene blue staining or electrical stimulation and recording. The tissues were fixed, dehydrated, embedded and examined in a manner identical to that previously found satisfactory for normal human specimens[1].

Definite changes in the fine structure of the myasthenic neuromuscular junctions were seen in all cases when compared with normal end plates of both adults and children subjected to thoracotomy for repair of cardiac defects. These changes may conveniently be classified into two major groups depending upon the extent and severity of the morphological changes:

Group I (Cases 1 and 2) show changes limited to the sarcolemma within the secondary synaptic folds. These changes consist of a peculiar focal loss of material from the normally uniform electron-dense portions of the sarcolemma resulting in focal areas of thinning. This conferred a "moth-eaten" appearance on nearly all secondary synaptic folds. No true "pores" have yet been demonstrated. Other elements of the neuromuscular junction essentially appeared unaltered.

Group II (Cases 3, 4 and 5) showed marked alterations in both presynaptic and postsynaptic components of the synapse consisting of a disorganized pattern of the secondary folds with coarsening and widening of these folds particularly in the distal portions. In addition to the focal loss of electron-dense material from the sarcolemma of the secondary folds, these folds seemed fewer in number and often "clubbed" in appearance. Decreased numbers of mitochondria in the sole plate sarcoplasm were often noted while the axon itself appeared separated from the primary cleft in some end plates.

In other end plates, oval, membrane-limited masses of moderately electron-dense material, often embedded in a granular matrix, were observed in the axoplasm. These "bodies" have not been further identified nor have they been described as yet in normal end plates.

Changes of these types were not observed in non-myasthenic neuromuscular junctions including biopsies showing less than optimum fixation. Acute changes in degenerating end plates following motor nerve section do not include alterations of the secondary clefts (3,4) although Reger (3) did observe widening of the primary synaptic clefts in denervated mouse neuromuscular junctions.

The first type (I) of abnormality found in myasthenic neuromuscular junction was observed in moderately severe chronic cases (Cases 1 and 2), and the second type (II) were seen in acutely ill or rapidly progressive cases (Cases 3, 4 and 5). While this small number of cases does not justify a definite correlation of fine structure abnormalities with the clinical status of the disease, our observations suggest that degeneration of end plates and subsequent repair may occur during the course of the exacerbations and remissions of the myasthenic syndrome. Further studies on the recently acquired sixth case and on biopsies from other myasthenics are contemplated in the future including biopsies from severely involved and uninvolved muscles from the same patient and before-and-after treatment biopsies on newly diagnosed cases.

b. Myasthenic Muscle Changes. Preliminary observations in some of these cases of myasthenia show fine structure changes in the muscle consisting of focal areas of loss of myofilaments, mitochondria of peculiar shape and distribution, and an increase in the granular (RNA) component of the cytoplasm in these changes has not been fully explored at present but it seems reasonable to speculate that they may represent atrophic changes secondary to the end plate changes.

2. Immune-Electron Microscopy and the Localization of Ferritin-labeled Botulinus Toxin in the Neuromuscular Junctions of Mice.

In an effort to devise a means by which the binding sites of chemical or bacteriological neurotoxins might be demonstrated a model system using ferritin-labeled immune serum to Staphylococcus aureus was investigated in collaboration with Major C. W. Smith and Major J. F. Metzger of the Bacteriology, Immunology and Infectious Disease Branch of the Armed Forces Institute of Pathology.

In this study (5) horse spleen ferritin was coupled to one isocyanate group of m-xylylene di-isocyanate by the use of a 0.5M carbonate-bicarbonate buffer, pH 9.0 at 4° C. The supernatant, the ferritin conjugate, was removed by centrifugation and subsequently coupled to an immune serum to Staphylococcus aureus, Type I, (ATCC 12598). The pre-immunization serum was similarly treated to serve as a control. Aliquots of a 10% buffered formalin fixed and washed suspension of homologous Staphylococcus organisms were allowed to react with both the immune conjugate and control conjugate. Both were washed, dehydrated in graded alcohol, and embedded in methacrylate with uranium acetate. Thin sections were cut and observed in the RCA EMU 3D electron microscope.

The ferritin antibody conjugate retained its specificity and the site of the antigen-antibody reaction was easily detected by visualization of the electron-dense ferritin micelles in electron photomicrographs. The ferritin-antibody conjugate was localized within a capsule-like material that surrounded each organism. The control material showed organisms enveloped with capsule-like material but without ferritin, the control conjugate having been completely washed from the background.

Localization of Botulinus Toxin.

Having demonstrated that ferritin-protein complexes can be made and specific sites of localization could be visualized electron microscopically, efforts were directed to the localization of botulinus toxin in the motor end plate area.

In a manner similar to that described for the preparation of ferritin-Staphylococcus antibody conjugate, horse spleen ferritin was conjugated to Botulinus neurotoxin B using m-xylylene di-isocyanate. A ferritin-protein control conjugate was also prepared in a similar manner. Three groups of mice were injected intraperitoneally; the first receiving the ferritin-toxin conjugate; the second, the ferritin-protein control conjugate, and the third, a suspension of nonconjugated ferritin. The ferritin-toxin conjugate retained its toxicity and at the point of complete paralysis, samples of intercostal muscle were prepared for electron microscopy as described earlier (1). At a comparable time interval following injection similar samples of intercostal muscle were obtained from both control groups of mice.

Methacrylate thin sections of the ferritin-protein conjugate and ferritin-control groups showed either no recognizable ferritin particles or only a few randomly

scattered particles when examined electron microscopically. No specific localization of these scattered particles was observed. In the ferritin-Botulinus injected animals, ferritin particles could be found in greater numbers and concentrated within the amorphous basement-membrane material. With some regularity ferritin micelles were observed within the sarcoplasm of the muscle although a consistent relationship of the iron micelles to any specific cytoplasmic structure is not yet apparent.

While these observations are tentative, more material is under study and should serve to verify these findings. The observations that have been made does suggest that Botulinus toxin is present at the synapse and is localized in highest concentration within the amorphous basement membrane material of the primary and secondary clefts at a time when paralysis of the neuromuscular system is well established.

3. Histochemical and Electron Microscopic Observations on Experimentally Produced Neuromuscular Atrophy and Degeneration.

Denervation Atrophy in Mice.

This experiment was undertaken to observe the acute and long term effects of axonal degeneration on the fine structure of mouse motor end plates and skeletal muscle cells.

The left femoral nerve of adult white mice was sectioned high upon the left psoas muscle and the distal end of the nerve retracted distally to prevent regeneration. A sham operation was carried out on the right side to serve as a control. Blocks of the upper third of the medial head of the vastus femorus muscle of both the denervated and control side were fixed for electron microscopy by immediate fixation of small blocks of tissue in White's Salt solution (0.5 os mol.) containing 1% osmium tetroxide, washing, dehydrating in graded alcohols and embedding in both methacrylate and Epon resins. Material was obtained from animals sacrificed at 1, 2, 4 and 7 days and 5 and 11 weeks after denervation. Some animals were sacrificed at the same time intervals to provide material for light microscopy, weight measurement to determine the degree of muscular atrophy and histochemical study of acetylcholinesterase distribution by the thiol acetic acid method.

End Plate Changes: Observations by electron microscopy on the acute stages of denervation degeneration of mouse motor end plates correspond closely with those made by Reger (3) on mice and by Birks et al (4) on the frog. At 2 days postdenervation no changes can be seen in the motor end plates.

By 4 days the axon filament within the snyaptic gutter show degeneration in some end plates. At 7 days axonal degeneration is complete in most end plates leaving a space where the axon should be and a widening of the primary synaptic cleft. No changes, however, are seen in any of the other components of the neuromuscular junction. In particular, with regard to the changes seen in the myasthenic end plates, the secondary folds and sarcolemma appear normal.

During the first week no change in the appearance of the end plate is noted histochemically when stained using the thiolacetic acid method. In fact only slight differences, i. e., slight irregularities in contour and width of the synaptic gutters and a tendency to clumping of the lead sulfide precipitate are observed even at 5 weeks post-denervation. No histochemical staining of end plates was seen at 11 weeks post-denervation.

End plate areas seen in the electron microscope after 5 weeks show the complete absence of any terminal axon filament but the secondary clefts persist in the sole plate area and except for the fact that they often open directly onto the bare surface of the muscle cell and seem somewhat irregularly distributed about what was once a synaptic gutter, they appear normal. The Schwann cell that normally "roofs" over the end plate area is usually greatly flattened with its cytoplasm stretched over the end plate area in long attenuated processes. The sole plate sarcoplasm is filled with mitochondria and Golgi profiles are prominent around some of the muscle nuclei.

Neuromuscular junctions in the 11 week animals have proved difficult to find at present because some identifying landmarks of end plate areas, such as small myelinated nerves and plump Schwann cells of characteristic appearance are no longer present. Search for these areas is continuing because the observations made thus far indicate that nerve damage leading to denervation of the end plate affects only the axonal component; the sarcolemmal folds, basement membrane, the sole plate sarcoplasm, the acetylcholinesterase activity and even the Schwann cell remain intact for a relatively long time. These findings may prove important in understanding reenervation of skeletal muscles following peripheral nerve injuries.

Denervation Skeletal Muscle Atrophy.

Concomitant electron microscopic observations of the muscle atrophy accompanying denervation were possible along with those of end plate degeneration. These are of interest because information about the fine structure changes of denervation muscular atrophy are scanty and because of the possibility of similarity to those changes seen in atrophic myasthenic muscles.

Gross weight measurements showed that no detectable atrophy had occurred in one week; 30 - 50% weight loss occurred in 5 weeks and 65-75% in 11 weeks when the denervated muscle was weighed and compared to the weight of its normal partner. Light microscopy showed that the atrophy was not uniform throughout the entire muscle. The most peripheral fasciculi showing the most advanced atrophy while the innermost showing the least. Blocks for electron microscopy were uniformly taken from the peripheral fasciculi at all stages in the process of atrophy.

Electron microscopic observations are as yet incomplete in most stages but some impressions are available particularly at 5 weeks post-denervation. At this stage in addition to the usual histologic findings of small fiber size, increased numbers of muscle nuclei with prominent nucleoli often arranged in a central position in the cell. There are interesting changes in the fine structure. A wide variation exists in the width of the arrays of myofilament with many appearing very thin and attenuated. No alterations in the size and arrangement in the individual myofilaments themselves have been observed. Many areas of loss of myofilaments are seen and these areas are filled with sarcoplasmic reticulin and granules interpreted as RNA protein. Numerous mitochondria of a great variety of sizes and shapes are dispersed in an irregular pattern throughout the arrays of myofilaments and are often concentrated around the muscle nuclei. Golgi profiles, difficult to find in mature muscle, are easily found most often in close vicinity to nuclei. No changes in the sarcolemma have been noted and there is no increase in width of the peripheral sarcoplasm. Occasionally moderately electron-dense membrane-limited oval or round bodies are seen among the mitochondria that often have an irregular scattering of every dense granules or membranes scattered irregularly throughout them. They resemble in many respects a body of unknown function called "microbodies" in other cells. The time sequence of these changes and their comparison with those seen in myasthenia are incomplete and await further study of the available material.

4. Histogenesis of the Neuromuscular Junction in the Developing Chick.

Observations made on the development of the motor end plate in the intercostal muscle of the chick by means of the thiol acetic acid method shows progression of development of end plates from a small flat disc at hatching to a circular structure at 1 week to a branched complex of synaptic gutters at 2 weeks similar to that seen in the mature mouse end plates. Examination of these regions with the electron microscope has begun but the observations are too fragmentary to discuss.

SUMMARY AND CONCLUSIONS:

The experience gained in this laboratory in applying the techniques of histochemistry and electron microscopy to the study of the motor end plate region in man and several animals has resulted in a better understanding of the anatomical fine structure of this region. These techniques have also been used to demonstrate a fine structure lesion in end plates from a neuromuscular disease; myasthenia gravis. This suggests that fine structure abnormalities may also be found in other pathological conditions in which the disease state is known only in terms of biochemical abnormalities or physiological dysfunction. Completion of the denervation experiments and the developmental study of the end plate should provide important information as to the manner of formation and degeneration of the neuromuscular junction and, as such, will form a basis for comparison with degenerative and regenerative changes occurring in other conditions.

This approach, plus the demonstration of the site of localization and action of a known neurotoxin, botulinus toxin, within the fine structure of the motor end plate, has made it possible to consider studies in the coming year of the sites of action or localization and the effect on fine structure of other chemical and biological agents of military interest that are suspected of acting in this area.

## REFERENCES


(1) Zacks, S. I. and Blumberg, J. M.: Observations on the fine structure and cytochemistry of mouse and human intercostal neuromuscular junctions.

(2) Osserman, K. E. (1958) Myasthenia Gravis. New York and London, Grune and Stratton.

(3) Reger, J. F. (1959) J. Ultrastruct. Res. 2, 269.

(4) Birks, R., Katz, B., and Wiledi, R. (1960) J. Physiol. (Lond.) 150, 145.

(5) Smith, C. W., Metzger, J. F., Zacks, S. I. and Kase, A. Proc. Soc. Exptl. Biol. and Med. (1960) 104, 336.


## PUBLICATIONS

During the Year - 1960-61

(1) Zacks, S.I., Bauer, W. C., and Blumberg, J. M. Letter to Nature. Abnormalities in the Fine Structure of the Neuromuscular Junction in Patients with Myasthenia Gravis. In Press.

(2) Zacks, S. I., Bauer, W. C., and Blumberg, J. M. The Fine Structure of the Myasthenic Neuromuscular Junction. J. Neuropath. Exptl Neuro. In Press.

(3) Smith, C. W., Metzger, J. F., Zacks, S. I., and Kase, A., Immune Electron Microscopy, Proc. Soc. Exptl. Biol. and Med. 4, 336-338 (1960)

(4) Zacks, S. I., Smith, C. W. and Metzger, J. F. Localization of Ferritin-Labeled Botulinus Toxin in the Neuromuscular Junction of Mice. In preparation.

## EXHIBITS

Material from the work of this project formed the basis for an exhibit entitled Normal and Pathologic Anatomy of the Neuromuscular Junction which was displayed at the 1960 meeting of the American College of Pathologists and American Society of Clinical Pathology in Chicago and at the Clinical Meeting of the American Medical Association held in Washington, D.C. 29 Nov-2 Dec 60, where it received an "honorable mention" award.

It was also exhibited at the 3rd International Congress of Clinical Pathology in Madrid, Spain; The International Academy of Pathology, London, England; the Association of Military Surgeons, Washington, D. C.; and presented as an exhibit at the U. S. Army Hospitals in Frankfort, Heidelburg, Landstuhl, Munich, and Wurzburg, Germany.

Material from the work of this project formed the basis for an exhibit entitled the Myasthenic Neuromuscular Junction which was displayed at the 1961 Annual Meeting of the American Medical Association held in New York City, New York, 25-30 June, where it received the Bronze Hoekten Award for scientific exhibits.

Conferences were held on this subject with Prof. Couteaux at the University of Paris, Prof. Coers, University of Brussels and Professor Wolfe at the University of London by Dr. Zacks and Colonel Blumberg.

# ANNUAL PROGRESS REPORT

## Title Page

PROJECT NO. 6X59-06-001 - Radiation and Thermal Burns

TASK NO. 4 Biological and biochemical effects of microwaves

NAME & ADDRESS OF REPORTING INSTALLATION:

Armed Forces Institute of Pathology

Washington 25, D. C.

NAME OF DEPARTMENT:

Office of the Scientific Director

PERIOD COVERED BY THE REPORT: 1 July 1960 - 30 June 1961

PROFESSIONAL AUTHORS OF THE REPORT:

Principal Investigator: R. E. Stowell, M.D.

Assistants: Bryce L. Munger, Capt., USAF, MC
Edward S. Reynolds,Jr.,Capt.,MC,USA
Vaman S. Waravdekar, Ph.D.

REPORTS CONTROL SYMBOL: RCS-MEDDH-288

SECURITY CLASSIFICATION: Unclassified

## ABSTRACT

PROJECT NO. 6X59-06-001 TITLE: Radiation and Thermal Burns

TASK NO. 4 TITLE: Biological and Biochemical Effects of Microwaves

NAME AND ADDRESS OF REPORTING INSTALLATION:

Armed Forces Institute of Pathology

Washington 25, D. C.

PERIOD COVERED BY THE REPORT: 1 July 1960 - 30 June 1961

AUTHORS:

R. E. Stowell, M.D.

Bryce L. Munger, Captain, USAF, MC

Edward S. Reynolds, Jr., Captain, MC, USA

Vaman S. Waravdekar, Ph.D.

REPORTS CONTROL SYMBOL: RCS-MEDDH-288

SECURITY CLASSIFICATION: Unclassified

### SUMMARY

The purpose of this research is to obtain fundamental information regarding the effects of pulsed microwaves on biological and biochemical systems. We are especially interested in the nonthermal effects of such energy on oriented molecular and cellular constituents.

Funds for this project first became available through the U.S.Army Medical Research and Development Command in March 1960. Because of the complex instrumentation involved, much time has been required awaiting delivery of equipment, assembling and testing components. Most, but not all, of the equipment on order has been delivered, so we have only recently been able to start the actual experimental work originally proposed. During the coming year the emphasis on this work will naturally shift from establishing of instrumental facilities to experiments. It is felt that the past year's work has been highly satisfactory and that significant experimental results should be attained during the coming year.

## BODY OF REPORT

PROJECT NO. 6X59-06-001 - Radiation and Thermal Burns

TASK NO. 4 Biological and biochemical effects of microwaves

DESCRIPTION:

Microwave Equipment

Components of the microwave generating apparatus shown in figure 1 have been acquired or contracted for during the period covered by this report. The individual components of this apparatus can be divided into six major divisions: Frequency generators, Pulsing systems, Low level simplifiers (=3 watts cw), High level simplifiers (>3 watts cw), Terminal load impedence balancing Pi networks, and Waveform monitoring oscilloscopes. Two frequency generators with a combined continuous range of 1 cps to 420 megacycles provide the broad spectrum of single frequencies at sufficiently narrow bandwidths (less than 0.01 per cent of generated frequency) for adequate study of discrete frequency effects. The pulsing system allows duty cycles of up to 35 per cent cw at all frequencies generated. (For the most part in our studies, duty cycles of 0.01 - 2 per cent are employed.) Low level amplification (= 3 watts cw) between 200 kc and 275 megacycles is performed by a cascade of five distributed amplifiers. Two amplifiers serve as high level amplifiers. Instruments for Industry Distributed Amplifier, Model 400, amplifies signals between 200 kc and 275 mc to 100 watts cw.

The HRB Singer amplifier amplifies 1-400 mc to 1 kilowatts cw. Output impedence of the terminal amplifier is balanced with the impedence of the sample load by a Pi bridge network consisting of variable parallel inductance and capacitance connected to the load through a fixed series inductance. Adjustment of parallel inductance and capacitance while viewing the waveform on an occilloscope at the load terminals allows precise impedence matching necessary for irradiation of samples with monochromatic microwave frequencies. Two oscilloscopes cover the frequency range between 200 kc and 400 mc. Actually the upper frequency range of one oscilloscope is in excess of 1 KMC.

Of all the equipment shown in Figure 1, only the HRB Singer High Level Amplifier has not been delivered. Lack of this equipment limits our studies to power levels to less than 100 watts cw. All other components in this diagram have been received, and integrated into the microwave generating apparatus which is functioning in our laboratory.

Sample Irradiation Cells

Two general types of cells have been developed for the irradiation of biological materials. The first, a glass microcell designed for direct microscopic viewing of the material being irradiated, is shown in figure 2. This cell, a modification of one developed in Dr. H. P. Schwann's laboratory at the University of Pennsylvania, consists of a glass slide, a 22X22 mm No. 1 1/2 glass coverslip, 10 to 60 micron platinum

wire and sealants. The height of the cell is determined by the diameter of the wire. The interwire distance can be accurately controlled. In typical operation, the signal is applied to the wires and the material examined by phase, interference, or polarization microscopy. Polarization microscopy has been used in our own studies with these cells. Similar preparations can also be employed in a refractometer. Typical field strengths range from 30 - 30,000 v/cm.

Cells for the irradiation of protein solutions, subcellular particulates, red blood cells and etc. are shown in figures 3 - 5. These cells constructed from plexiglass tubing, platinum foil, and platinum wire, are of syringe construction with provision for circulation of water as coolant in a jacket about the barrel and in the plunger (all but the 1/4" cell.) Platinum foil electrodes, one placed on the bottom face of the barrel, the other on the face of the plunger form a plate condensor type irradiation cell, the interelectrode distance of which is variable from but a fraction of a millimeter to 2 - 3 centimeters. Temperature is measured in these cells by insertion of a copper constantan thermocouple through an inlet into the sample chamber to a point where it lies at, or near the ground plate. Such variable volume cells allow irradiation of samples from 0.01 - 10 cc volume under essentially similar conditions in a closed temperature controlled space. Following irradiation, these samples can be readily retrieved and studied without altering the geometry of the cell in any way.

The effects of relative capacitive reactance and resistance on the solution being irradiated can be examined with these cells. For example, at an interelectrode distance of 1 millimeter the resistance offered by the 1/4" cell is 16X that by the 1" cell and the capacitive reactance 16X less. Although varying cell geometry will not alter specific resistivity, or unit dielectric properties of the solution, phase relationships between applied RF voltage and power transmitted to the solution being irradiated will be markedly affected.

## Experimental Studies of Birefringence in Microwave Fields

Experimental studies with the visual cell have centered on studying the optical effects seen in liquids under the influence of microwave energy. Since colloidal particles orient in a microwave field (pearl-chains), large molecules which possess axial asymmetry might be expected to orient in linear arrays and thereby demonstrate birefringence. However, solutions of water, glycerin, alcohols of all types, fatty acids, etc., become strongly birefringent when placed in a microwave field in the cell described (figure 2). A component of this birefringence has been demonstrated to be due to heat-induced strain birefringence of the glass slide and coverslip. The physical parameters of the system obey the laws for power absorption with the intensity of the birefringence being proportional to the frequency, dielectric properties of the medium, square of applied field intensity, viscosity and conductivity.

Accurate measure of the magnitude of birefringence is possible using a photoelectric cell to measure the intensity of the elliptically polarized light. The sign of the birefringence under these conditions is positive with respect to the axis of RF conduction. The threshold for pearl-chain formation of colloidal particles (polystyrene spheres, red blood cells, and colloidal silver in solutions of water, alcohol, and glycerin) is identical to the threshold for detectable birefringence. Thus the power absorption by the solution produces both effects at the same field strength.

In further studies of birefringence of microscope preparations in microwave fields, the problem of heat-induced strain birefringence of the glass slide and coverslip has been obviated by the location of a type and source of glass which does not become strain birefringent when heated. This glass, which consists of approximately 70% PbO, is being made for us at the National Bureau of Standards. Following casting of a satisfactory slug of glass, it will be cut into coverslips and slides for use.

A preliminary account of this material has been presented at the meeting of the Biophysical Society, St. Louis, Mo., February 16, 1961.

Irradiation Studies on Enzyme Proteins.

The purpose of this study is to ascertain (1) if enzyme proteins can be altered by high energy pulsed microwave irradiation under isothermal conditions using altered enzyme activity

as indicator, (2) if the anticipated alterations in enzyme activity are associated with changes in structure indicated by altered ultracentrifugation pattern, and (3) to determine if all proteins studied are affected at the same frequency, irrespective of molecular weight, or size, or if microwave effects are frequency specific for each protein. The ultimate goal of this experimentation is more precise knowledge of nature of interaction of microwave energies with macromolecules. The 1 - 40 megacycle zone of microwave energies has been chosen for initial study for two reasons: It is the zone of beta-dielectric dispersion for proteins, and Colonel Sven Bach of Fort Knox has had apparent success dissociating gammaglobulin in this range of the microwave spectrum.

These studies on the effects of microwave irradiation on enzyme proteins have been delayed by equipment failures in the high level amplifier systems. Now that many of these problems have been corrected, solutions of Yeast Alcohol Dehydrogenase (MW = 150,000), have been irradiated at intervals from 1 - 40 megacycles and the irradiated enzyme solutions assayed for loss of activity -- loss of activity being employed as a readily available indicator of microwave damage to enzyme protein. To date over 200 irradiations of Yeast Alcohol Dehydrogenase have been made in this range, most in the range from 1 - 10 megacycles. In this latter range decreases in activity are noted

between 2 - 4 magacycles at 7-9°C at 100 volt/cm gradients. In some instances the activities of irradiated enzymes have decreased to below 1/10 the control value. These results, though repeatable in this range, are difficult to exactly reproduce at a given frequency due to temperature variation during the irradiation, i.e., 3 - 5° C rises during the experiment. In order to eliminate or greatly reduce the magnitude of this problem of temperature variation, lower field voltage gradients of 50 volts/cm. are now being used. Pulsed high voltages will also be used following the delivery of the 1 kilowatt amplifier.

Following determination of the molecular dissociation frequencies for Yeast Alcohol Dehydrogenase at 4°C between 1 and 4 megacycles, Molecular dissociation frequencies for Horse Liver Alcohol Dehydrogenase (MW 73,000), Glutamic Dehydrogenase (MW 1,000,000), Lactic Dehydrogenase (100,000) and Pancreatic Ribonuclease (15,000) will be determined under the same conditions. Thus it may be possible to ascertain whether molecular size, and/or susceptible peptide linkages influence enzyme denaturation at these frequencies.

These enzymes chosen are highly purified crystalline proteins of different molecular weights and size. Samples of enzymes to be irradiated are either monodisperse upon ultracentrifugation or have single dominant peaks.

# BLOCK DIAGRAM OF 1 KILOWATT CONTINUOUS WAVE OR PULSED 1-400 MEGACYCLE RF GENERATING NETWORK

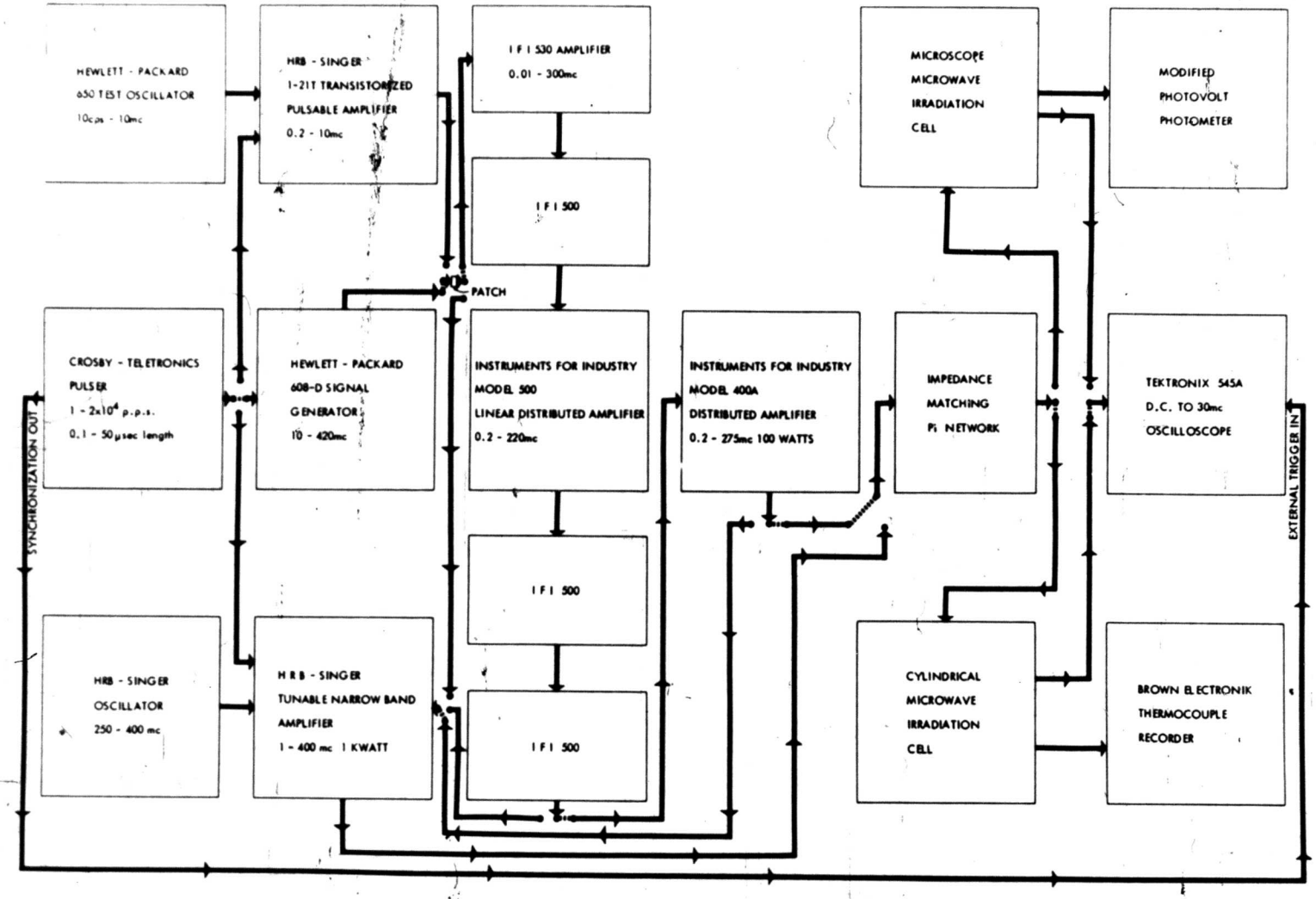
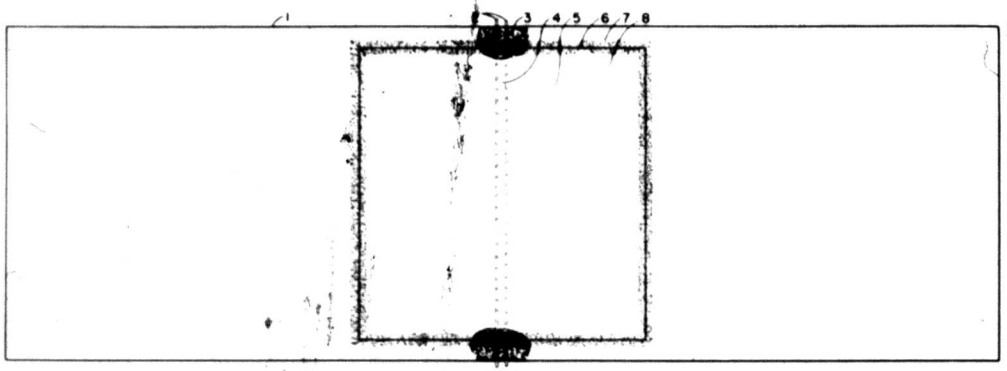
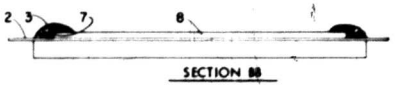
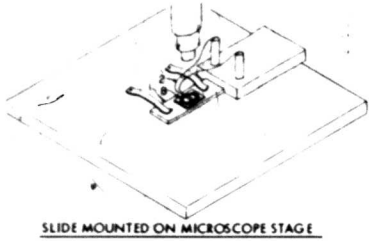



Fig. 1

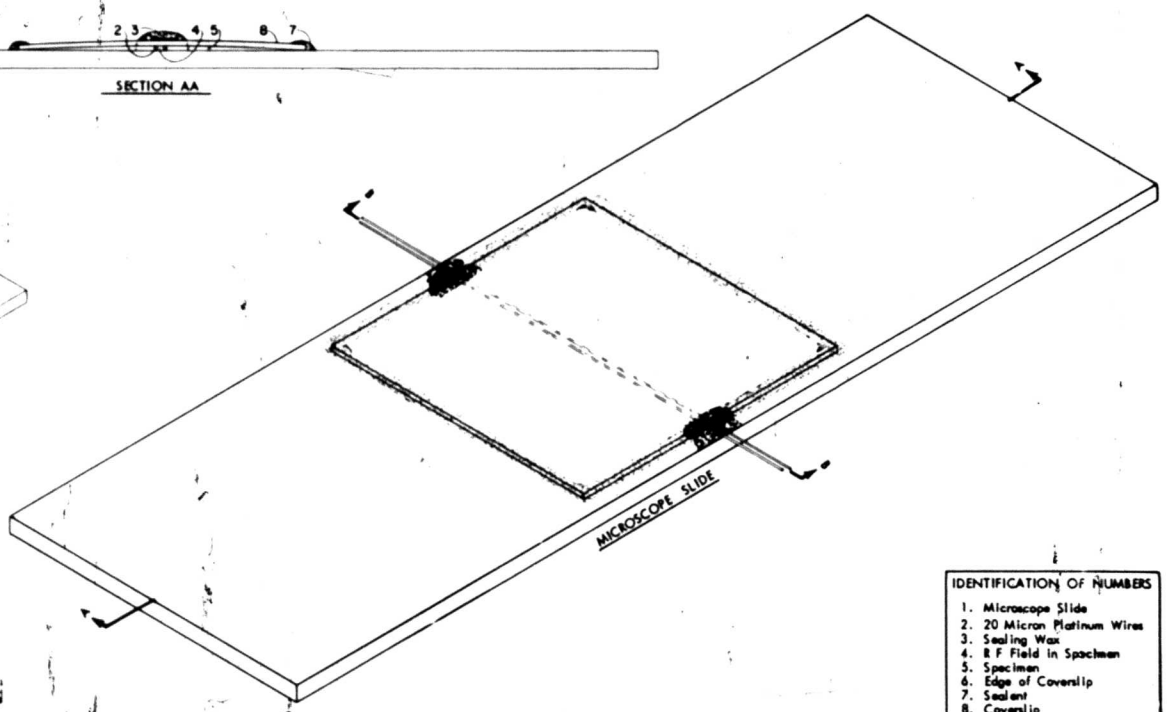
# MICROSCOPE MICROWAVE IRRADIATION CELL



SLIDE MOUNTED ON MICROSCOPE STAGE



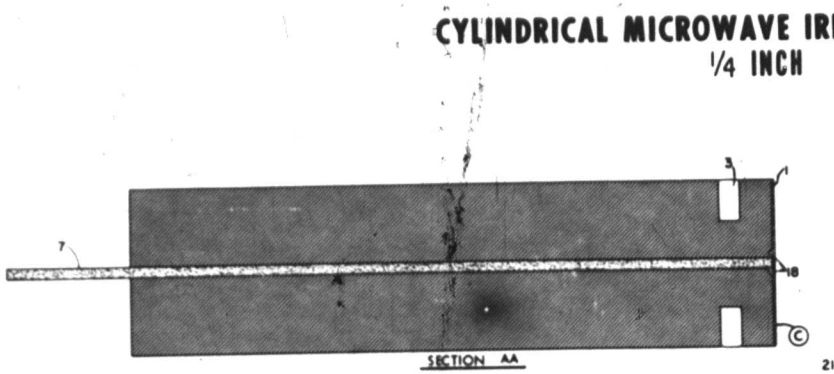

IDENTIFICATION OF NUMBERS

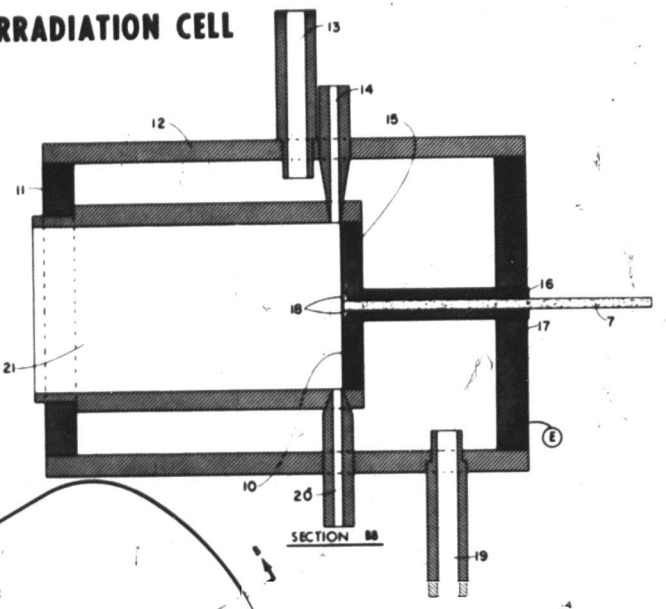
1. Microscope Slide
2. 20 Micron Platinum Wires
3. Sealing Wax
4. R F Field in Spacimen
5. Specimen
6. Edge of Coverslip
7. Sealent
8. Coverslip

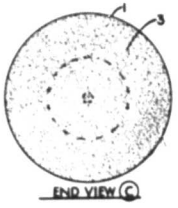

AFIP

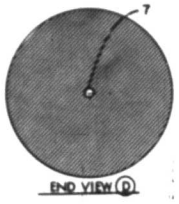
Fig. 2

# CYLINDRICAL MICROWAVE IRRADIATION CELL
## 1/4 INCH



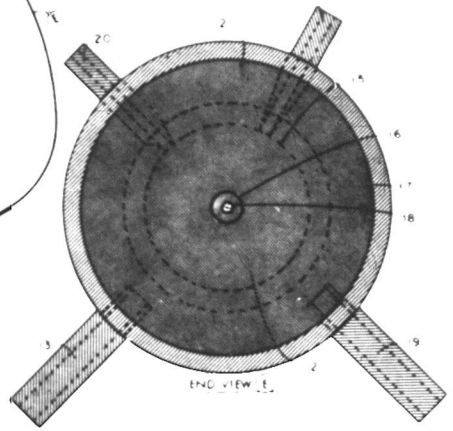









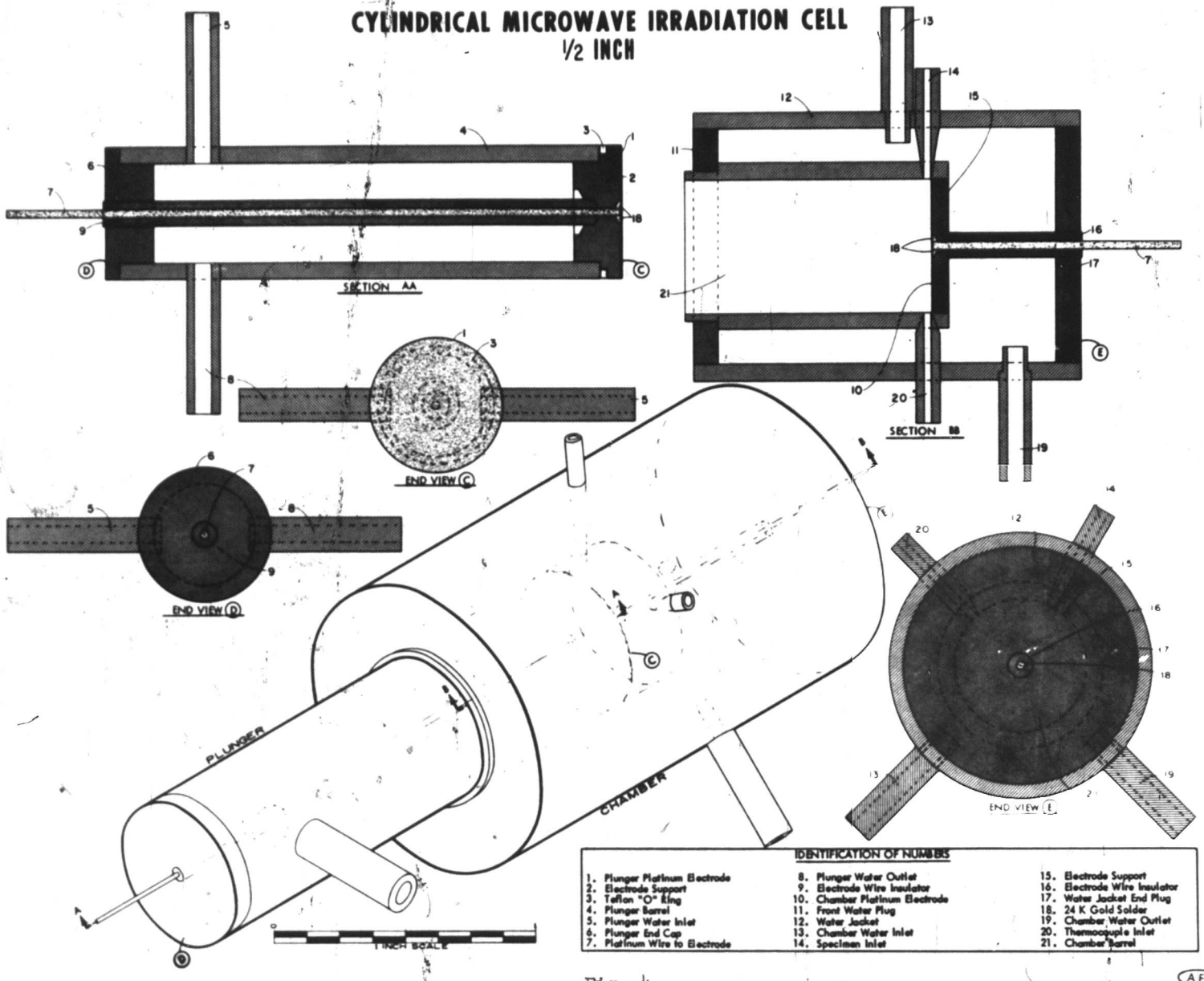

**IDENTIFICATION OF NUMBERS**

1. Plunger Platinum Electrode
2. Electrode Support
3. Teflon "O" Ring
4. Plunger Barrel
5. Plunger Water Inlet
6. Plunger End Cap
7. Platinum Wire to Electrode
8. Plunger Water Outlet
9. Electrode Wire Insulator
10. Chamber Platinum Electrode
11. Front Water Plug
12. Water Jacket
13. Chamber Water Inlet
14. Specimen Inlet
15. Electrode Support
16. Electrode Wire Insulator
17. Water Jacket End Plug
18. 24 K Gold Solder
19. Chamber Water Outlet
20. Thermocouple Inlet
21. Chamber Barrel

Fig. 3

AFIP

# CYLINDRICAL MICROWAVE IRRADIATION CELL
## 1/2 INCH




**IDENTIFICATION OF NUMBERS**

| | | |
|---|---|---|
| 1. Plunger Platinum Electrode | 8. Plunger Water Outlet | 15. Electrode Support |
| 2. Electrode Support | 9. Electrode Wire Insulator | 16. Electrode Wire Insulator |
| 3. Teflon "O" Ring | 10. Chamber Platinum Electrode | 17. Water Jacket End Plug |
| 4. Plunger Barrel | 11. Front Water Plug | 18. 24 K Gold Solder |
| 5. Plunger Water Inlet | 12. Water Jacket | 19. Chamber Water Outlet |
| 6. Plunger End Cap | 13. Chamber Water Inlet | 20. Thermocouple Inlet |
| 7. Platinum Wire to Electrode | 14. Specimen Inlet | 21. Chamber Barrel |

Fig. 4

AFIP

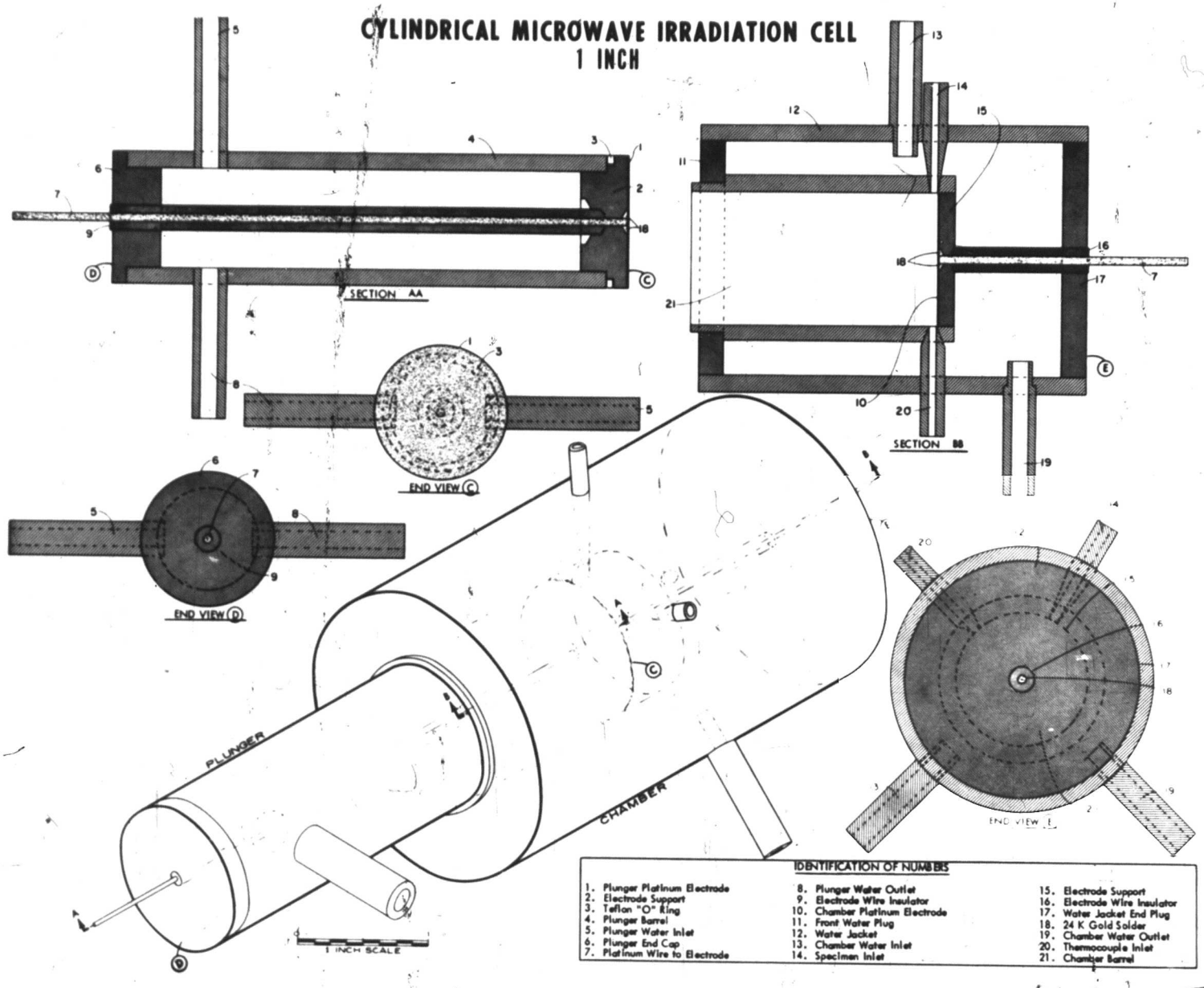

CYLINDRICAL MICROWAVE IRRADIATION CELL
1 INCH
SECTION AA
SECTION BB
END VIEW C
END VIEW D
END VIEW E
PLUNGER
CHAMBER
1 INCH SCALE
IDENTIFICATION OF NUMBERS
1. Plunger Platinum Electrode
2. Electrode Support
3. Teflon "O" Ring
4. Plunger Barrel
5. Plunger Water Inlet
6. Plunger End Cap
7. Platinum Wire to Electrode
8. Plunger Water Outlet
9. Electrode Wire Insulator
10. Chamber Platinum Electrode
11. Front Water Plug
12. Water Jacket
13. Chamber Water Inlet
14. Specimen Inlet
15. Electrode Support
16. Electrode Wire Insulator
17. Water Jacket End Plug
18. 24 K Gold Solder
19. Chamber Water Outlet
20. Thermocouple Inlet
21. Chamber Barrel

Fig. 5

ANNUAL PROGRESS REPORT

Title Page

Project No. 6X61-03-001 - Procedures for Quantitative Electron Microscopy

Task No. 5 Photometric Procedure for Weight Determination of Submicroscopic Particles

Name and Address of Reporting Installation:

Armed Forces Institute of Pathology

Washington, D. C.

Name of Department and Division:

Department of Pathology

Division of Basic Sciences

Biophysics Branch

Period Covered by the Report: 1 July 1960 - 30 June 1961

Professional Authors of the Report:

Principal Investigator: G. F. Bahr, M. D.

Assistant: Elmar H. Zeitler, Ph.D.

Reports Control Symbol: (RCS-MEDDH-288)

Security Classification: (Unclassified)

## ABSTRACT

Project No. 6X61-03-001 Title: Procedures for Quantitative Electron Microscopy

Task: Photometric Procedure for Weight Determination of Submicroscopic Particles

Name and Address of Reporting Installation:

Armed Forces Institute of Pathology

Washington, D. C.

Period Covered by the Report:

1 July 1960 - 30 June 1961

Authors:

G. F. Bahr, M. D. and E. H. Zeitler, Ph.D.

Reports Control Symbol: (RCS-MEDDH-288)

Security Classification: Unclassified

## SUMMARY

The purpose of this research project has been and will be in the years to come, to provide the practical Electron Microscopist with methods for the determination of the mass and composition of small biological objects well beyond the range of any other technique for mass (dry weight) determination.

A photometric procedure for rapid determination of weight of isolated particles down to a size of 200 Å is described. Under

Project No. 6X61-03-001

Task: Photometric Procedure for Weight Determination of Submicroscopic Particles

normal conditions of electron microscopy, weights of down to $10^{-18}$ g can be determined with an inaccuracy of less than 10%. By lowering the accuracy or using more elaborate measures (such as very low accelerating voltage) one to two orders of magnitude for the lower weight limit can be gained.

The method can now be applied to population studies of biologic particles, especially those of inhomogeneous and odd-shaped entities. Individual losses of matter through the action of enzymes and the uptake of specific stains can be measured quantitatively. The method extends the possibilities of individual mass-weight determination to the biologically important region of submicroscopic particles.

## BODY OF REPORT

Project No. 6X61-03-001 Procedures for Quantitative Electron Microscopy

Description:

The photographic density of an electron micrograph is an equivalent to the mass distribution in the object.

The task of the first phase of the project was to develop and approve a procedure yielding immediately the total dry mass by means of simple optical measurements on these micrographs.

It is needless to emphasize the importance of the weight of single submicroscopic particles. All other published methods give average values of such particle populations only.

Progress:

The work has proceeded along the following steps:

A) A thorough investigation of the relationship between the weight of a given object and its photographically recorded image. Well-defined model systems were used in order to determine experimentally the influence of the working data of the electron microscope, (aperture, high voltage, focus) and the factors involved in the photographic processing as well (exposure time, developer, developing time, developing temperature, photographic material).

B) The establishment of accurate, handy and quick photometric procedures for quantitative electron microscopy.

C) Standardization of exposure.

D) Calibration procedures for electron microscopes by means of external standards, and

E) The evaluation of populations of biological objects.

A. It has been found in earlier studies that the contrast of an electron microscopic object in a micrograph is a direct measure of the transradiated mass per unit area[1]. The pertinent theoretical derivations have been experimentally confirmed. This fact offers the possibility of determining the total weight of any object by measuring its contrast, point by point (scanning).

The scanning technique is, however, seriously limited in being rather complicated, cumbersome and time consuming. It requires more and more scanning tracings in direct relation to the number of inhomogeneous and odd-shaped objects. The small number of objects which can thus be processed cannot reveal any biological significance due to statistical variations. For the measurement of isolated small biological entities and subcellular units, a photometric procedure has now been developed which yields directly an integrated value of the dry weight of the object. The point-wise (differential) evaluation with a subsequent arithmetical summation is achieved by optical means in one step. The photometric procedure is described in more detail in Section B.

Ideal model objects are spherical latex particles in the range of 0.08 to 0.26 microns diameter. Suspensions of such spheres have become available in recent years. Their advantages are:

1. Remarkable uniformity in shape and size.
2. Their electron-scattering properties are like those of biological material (low atomic number).
3. For a given magnification the diameter can be determined on the micrographs. Thus the weight can be calculated from geometrical measurements only and compared with the values read on the photometer.
4. The argument under 3 holds also for clusters of latex spheres, which represent an ideal inhomogeneous object.

The study of these spheres proved the theoretical considerations to full satisfaction:

After checking the applied magnifications carefully by means of standardized gratings and measuring the diameter of the particles, their weights have been calculated. The photometric measures and the calculated weights coincide. Consequently, it is hereby demonstrated that the dry weight of any inhomogeneous object can be derived from its density in the electron micrograph. The introduction of the specific gravity of the object yields a value for the volume.

The various parameters in exposing, developing, and evaluating the micrograph have been studied to furnish the Electron Microscopist with a survey of the whole field and to enable him to choose the most suitable set of working data for his particular problem. A more detailed description is in press[2].

B. A special photometer has been designed which allows the determination of the weight of a particle from its electron micrograph by a single measurement.

The principle of the photometer is based on the fact that the optical transmission of a photographic plate can be used for an optically performed area integration. A telecentric lens system depicts a light source on the sensitive area of a detector. If a photographic plate is inserted in the optical path of this system, the light intensity of a lamp on the detector changes in proportion to the integrated transmission over the transluminated area.

The photometer used in our previous studies was optically improved. Devices were incorporated in the optical system to reduce the glare. A head-on-type photomultiplier had to be installed (instead of the regular-type photomultiplier). All the electrical supplies had to be stabilized due to the considerable variations of the 115 AC line. A high voltage power supply with extreme stabilization was designed and built. Also, the lamp

supply is obtained now from a new, highly stabilized and fully transistorized power supply. For easy reading of large numbers of measuring values, a digital voltmeter was acquired and installed in combination with a printing calculating machine. The reading from this voltmeter is immediately multiplied by the appropriate instrumental factor, and the permanent printed record facilitates filing and further processing of the data. The above measures tripled the working capacity of the photometer.

C. The photometric procedure requires standardized exposure. To achieve this, a precise shutter-timer system and a stabilized electronic exposure meter (photomultiplier) have been designed and attached to the Siemens Electron Microscope in use.

D. The general applicability of the described method requires more than one standardization. Instead of calculating the weight indirectly from the density as in the case of latex spheres, it is desirable to weigh external standards on a macroscopic balance. A procedure for this employs Formvar films thin enough (200-300Å thick) to be used in the electron microscope and large (8 x 10 cm) and homogeneous enough to obtain gravimetrical reliable measures of their weight per area. They are cast on both surfaces of a very even glass slide; one side is scored with a knife, and when the slide is dipped into water, the scored strips of film float onto the water surface. The strips are then picked up with the usual electron microscopic object grids, so that they build up to a step wedge. The film of known

area on the other side of the slide is dried and its weight determined. The available balances, however, were too insensitive for this procedure. Therefore, a vacuum balance of the torsion-counterbalance type had to be built. This instrument is suitable for a weight range of up to 1000 gamma and can be read with an accuracy of $\pm$ 1 gamma. Now the contrast produced by the step wedge in the electron microscopic image can accurately be correlated to the weight per unit area, producing this contrast.

E. In accordance with the aims of this research project, pilot experiments were undertaken to elaborate new techniques and to check existing techniques for the preparation of biological material suitable for quantitative electron microscopy. Mitochondria were chosen as very interesting objects for quantitative population analysis. Thus the capabilities of the technique could be demonstrated practically.

Summary and Conclusions:

A photometric procedure which makes possible the determination of the weight of submicroscopic particles down to $10^{-18}$ gm is now available.

We are now in the process of applying this method to biological problems.

For the other part of the project which deals with the changes in electron microscopic objects due to irradiation in the electron beam, the photometer is an important aid.

List of Publications:

1. Zeitler, E. and Bahr, G. F.: The interpretation of contrast in an electron micrograph. Symposium on contrast in electron microscopy, Santa Monica 1958 publication in RCA Scientific Instruments News, pp. 7-15, 1960.
2. Zeitler, E. and Bahr, G. F.: J. Appl. Phys. A Photometric Procedure for Weight Determination of Submicroscopic Particles. (In Press)

# ANNUAL PROGRESS REPORT

Project No. 6 x 60-01-001 - Internal Medicine

Task No. - Investigation of Respiratory Diseases of Laboratory Animals

Name and Address of Reporting Installation:

Armed Forces Institute of Pathology, Washington 25, D. C.

Name of Department and Division:

Virology Branch, Veterinary Division

Period Covered by the Report: 1 July 1960 - 30 June 1961

Professional Authors of the Report:

Principal Investigator: Fred D. Maurer, Col., VC, USA

Assistants: Robert A. Crandell, Maj., USAF, VC, James R. Ganaway, Capt., USAF, VC, P. H. Craig, Capt., USAF, VC, J. L. Adcock, Capt., VC, USA, W. H. Niemann, Lt., VC, USA, D. F. Hersey, Capt., USAF (MSC)

Reports Control Symbol: RCS-MEDDH-288

Security Classification:

Unclassified

ABSTRACT

Project No. 6 x 60-01-001 Title: Internal Medicine

Task No. 6 Title: Investigation of Respiratory Diseases of Laboratory Animals

Name and Address of Reporting Installation: Armed Forces Institute of Pathology, Washington 25, D. C.

Period Covered by the Report: 1 July 1960 - 30 June 1961

Authors: Fred D. Maurer, Col., VC, USA, Robert A. Crandell, Maj., USAF, VC, James R. Ganaway, Capt., USAF, VC, P. H. Craig, Capt., USAF, VC, J. L. Adcock, Capt., VC, USA, W. H. Niemann, Lt., VC, USA, D. F. Hersey, Capt., USAF (MSC)

Reports Control Symbol: RCS-MEDDH-288

Security Classification: Unclassified

SUMMARY

It is the objective of this research program to provide basic insight and information on the nature, prevention and control of some of the major respiratory diseases of laboratory animals. The principal area of study has been around respiratory diseases in cats and rats because of the prevalence of respiratory diseases in these species and their importance as experimental animals in numerous research projects. Considerable progress has been made during the current year. At least two respiratory diseases

in rats have been extensively studied both from a pathological standpoint and by characterization of the agents involved. Studies on the feline viruses associated with the respiratory tract of cats have been expanded and considerable progress made in characterizing the agents. The immunological classifications of a large number of these viruses have been made by both complement fixation and serum neutralization tests. A large portion of the work carried out under this grant has been published in professional journals and a number of other papers have either been submitted or are in preparation for submission to such journals for publication.

BODY OF REPORT

Project No. 6 x 60-01-001 Title: Internal Medicine

Task No. 6 Title: Investigation of Respiratory Diseases of Laboratory Animals

Description: Feline Viruses

Accurate work involving laboratory animals requires a healthy animal free from intercurrent infections and contaminants. To this end our attention has centered around attempts to gain information on the nature, accurate diagnosis, prevention and control of many of the most important diseases of laboratory animals. The present study has confined itself to the study of respiratory diseases encountered in rabbits, rats, mice and cats.

Progress:

Considerable progress has been made in this area of the research program. Studies have been completed and published on two feline viral diseases, feline viral rhinotracheitis associated with a virus designated as FVR, and another feline respiratory disease associated with a viral agent designated as CFI or FIV. Experimental transmission studies have been described for both these agents. Biophysical characterization of one of these agents has been completed and electromicrographs obtained. This paper is in preparation for publication. Biophysical studies (ultracentrifugation, electromicrography, etc.) are currently in progress for the FIV virus. Preliminary attempts to relate the feline virus to certain known human agents have been unsuccessful. (See publication on this subject.) Further studies in this area are being carried out using additional agents. The utilization of feline renal cell tissue cultures for the growth of viral agents other than feline has been investigated and a number of other viruses found to propagate readily in such cells. (See publication on this subject.) Considerable progress

has been made in the serological classification of a large number of feline viruses so far isolated. These studies have included both complement fixation and serum neutralization tests. Results of such studies have indicated a common group antigen in a number of these feline viral isolates when tested by complement fixation (CF). To assist in the preparation of improved CF antigens, attempts have been made to grow feline viruses in a tissue culture system other than feline kidney. To date the most promising results have been obtained with hamster kidney cells. These studies are being expanded as time permits. A number of feline viruses shown to be related by complement fixation testing are immunologically distinct when tested by serum neutralization tests in feline kidney. (Manuscript submitted for publication.) The relationship of the more recently isolated feline viruses (isolated in this laboratory) to a number of the older more established feline viruses (feline pneumonitis, panleukopenia virus, etc.) is currently being studied. A cytochemical study of one of the feline viruses has been completed (manuscript submitted for publication) and additional studies with fluorescent antibody, acridine orange fluorescent microscopy and electron microscopy are being carried out with a selected number of the other feline viruses which are characterized by a different cytological behavior than that demonstrated by the FVR virus already studied. Studies with UV light irradiation have indicated that FVR virus can be quickly inactivated and made non-infectious. Use of such material for immunization purposes is soon to get underway. The installation and testing of new isolation cages in the Virology Branch now make it possible to carry out transmission studies on a selected number of feline viruses. These studies are scheduled for the next several months.

A series of animals have been inoculated with aerosols of selected

viruses--FRI-1-61, CSF and FRI-5-61--all of which gave some earlier indication of pathogenicity for the cat. Records were kept on the clinical symptoms of all animals, including daily temperature recordings. Blood samples were taken before and three weeks following inoculation for serological studies by both complement fixation and serum neutralization studies. All animals were autopsied three weeks post inoculation and tissues processed for microscopic examination. The results of the histopathology study are not available nor are the data on the serological response of these animals completed. The completed data will be compiled and analyzed to determine the significance of additional studies which might be performed.

Chronic Murine Pneumonia

During the past year much insight into the problem of this disease complex has been obtained. The greatest obstacle which confronted us at the time of the last report was the acquisition of, and holding for extended periods of time, rats, which by all known measures, could be considered "clean" or free of this complex. A battery of isolation cages which utilize filtered air was installed. Specific pathogen-free albino rat weanlings (from the Walter Reed Army Institute of Research colony) were placed in these cages and given a chemotherapeutic agent reported by other workers to exert a beneficial effect in prevention of this disease (Habermann and MacPherson, NIH, personal communication). These rats have been raised in such an environment to ages in excess of one year with most encouraging results. The administration of the chemotherapeutic agent for extended periods to these rats appears to exert no deleterious effect on their growth rate or fecundity. As yardsticks by which we establish the clean status of these animals, we can say that: (1) no gross or histologic lesions have been

observed in any of these animals (approximately 24 complete autopsies of varying ages through one year). (2) Culture of lung suspensions from these rats on blood agar, in thioglycolate media and on enriched agar for PPLO isolation has failed to reveal the isolation of a single culturable organism. To preclude the possibility that these results might have been influenced by a static effect of the chemotherapeutic agent, it was withheld from a group of rats for a period of one week and a like bacterial determination made with the same negative result as before. (3) The intranasal instillation of rat lung homogenate suspensions from these rats into specific pathogen-free mice (WRAIR colony) revealed no evidence of illness during a two-week observation period and showed no gross lung pathology at the time of sacrifice. (Serial passage of normal mouse lung suspensions in mice from this colony on several occasions has failed to reveal any evidence of latent viral respiratory pathogens in these mice.)

The microscopic lesion most often observed in "diseased" rats is a lymphoid hyperplasia which progresses to the extent that large bands of mononuclear cells can be seen on a stained slide preparation (even macroscopically) surrounding the major bronchi and often forming cuffs around the blood vessels. A bacterial agent has routinely been isolated from infected rats of all ages from the NIH and WRAIR open colonies which will induce a similar type lesion in clean rats. That this agent is readily isolated from rats of all ages lends support to the concept of a carrier state and a chronic infection which is in keeping with the description of this disease complex. Experiments are in progress which have been designed to establish the validity of this concept. Reports by numerous investigators on an antigenically related organism suggest that this organism may possess the capability of exerting an adjuvant effect and an ability to decrease host resistance to agents otherwise considered

as non-pathogens so that infection results. Experiments designed to test this hypothesis are also in progress. If the organism employed in these studies should possess such properties and a carrier state exists, a more precise explanation for the multiplicity of pathological findings reported in the literature as existing in aged rats in various colonies would be available.

To determine whether this bacterial pathogen was ubiquitously present in other colonies throughout the United States, a sample survey was conducted of six colonies (5 commercial, 1 university) representing widely dispersed geographical areas (north, south, both coastal regions and the midwest). These rats, twelve from each source, were subjected to a series of tests to determine the following information:

1. The incidence of the bacterial agent most commonly associated with chronic murine pneumonia in this laboratory.
2. The presence of other bacteria and the frequency with which they occurred.
3. Incidence of PPLO isolation (from lung suspensions only).
4. Incidence of non-bacterial (viral?) agents based on the development of characteristic lung lesions in animals according to the technique described by Nelson.
5. Incidence of otitis media.
6. The correlation in gross and microscopic pathology (complete autopsy and work-up on all parenchymatous organs) with the above findings.
7. Demonstration of serologic response using serum collected from these rats against the agents isolated.

The findings of this survey are not complete to date but a partial result follows:

| Colony | Isolation of Organism Identical to that most Commonly Isolated from Indigenous Rat Colony | *Isolation of Virus-like Agent | Incidence of Otitis Media - Bilateral or Unilateral |
|---|---|---|---|
| A | **0/12 | 0/12 | 2/12 |
| B | 0/12 | 12/12 | 0/12 |
| C | 12/12 | 12/12 | 9/12 |
| D | 0/12 | 0/12 | 9/12 |
| E | 10/11 | 10/11 | 8/11 |
| F | 6/9 | 0/9 | 9/9 |

*The term "virus-like agent" refers to the production of uniform lesions in lungs of intranasally inoculated mice which are not attributable to bacteria (Nelson).

**Ratio of positive isolations to the number of rats examined.

No other bacterial agent was isolated with any frequency to suggest its role in chronic disease.

As regards the virus-like agent(s) isolated, data are accumulating which tend to indicate that all isolates are either identical or closely related. The clinical character and gross pathology of the disease produced in the inoculated mice were indistinguishable within each group and between groups. The characterization of this virus-like agent, as well as attempts to isolate and propagate it in tissue culture and/or embryonated eggs, is currently in progress.

One isolate of this agent was passed blindly in hamster kidney tissue culture. At the second passage, a poorly defined cytopathogenic effect (CPE) was observed. Now in the sixth passage, CPE is regularly produced between 48 and 72 hours. Infected cells stained by the May-Grunwald Giemsa method reveal numerous cocco-bacillary bodies both intra- and extra-cellular. Though unable to initially cultivate this organism on Barille's PPLO agar, attempts are now being made to grow it in enriched broth media. Early results are encouraging.

This same isolate was passed blindly via the yolk sac route in 6-day embryonated eggs. Deaths occurred as early as day 6 post inoculation. After 6 such passages, deaths occurred as early as day 4 with 100% mortality by day 6. That the same agent has been grown in both hamster kidney tissue culture and embryonated eggs is suggested by the finding that hamster kidney cultures inoculated with infected yolk sac suspension produce the same CPE and cocco-bacillary bodies previously described. Passage of the agent isolated in these two host systems to the mouse is currently in progress. In addition, serological studies on sera of both naturally infected and artificially immunized animals, using tissue culture and yolk sac antigens, are currently in progress.

The microscopic pathology is currently being carried out. The incidence of gross pathology is in keeping with the published results of Innes, et al.

Thus far our findings indicate that the known bacterial agent and the virus-like agent (PPLO?) are most frequently found in the samples tested. In those colonies where they are found, they are present in virtually 100% of the rats. There are colonies from which neither of these agents was isolated, but it is expected that antibiotics and/or chemotherapeutic agents are responsible for this negative result since there was no significantly different finding in the gross pathology of lungs between groups (with the exception of the University colony, which supplied animals which were thought to be most severely affected).

The transmission into clean rats using the two most frequently isolated agents described above, their characteristics, antibiotic sensitivity spectrum and other means of prevention or control in colonies, and their relationship to other known pathogens are being investigated.

## Snuffles in the Rabbit

Six female adult rabbits, free from clinical signs of snuffles, were housed and bred in the building. Eighteen days later the now gravid females exhibited the symptoms compatible with "snuffles". Temperature, white blood counts, differential counts, habitus, bacterial isolations, serum titers and histopathology were determined and recorded. A total of six females and young were examined; the young were sacrificed at weekly intervals in order to obtain an estimate of the time sequence effect on the tissues, frequency of isolation and serum titer.

Clinical Symptoms: The presence or absence of nasal discharge and its character could not be predicted from day to day. Some animals showed a consistent nasal discharge while others showed intermittant discharge or none at all. Appetite and elimination were considered to be normal. Temperatures were found to be slightly higher than normal with some peaking at 104° F+. A rather consistent conjunctivitis was associated with some of these animals. On isolation attempts no Pasteurella or Bordetella were recovered. A relative increase was seen in the circulating white blood cells with the majority of animals peaking as high as 20,000 total WBC and dropping as low as 6,000.

Bacterial Isolations: The predominant organism recovered from nasal washings was Bordetella bronchiseptica. Pasteurella was isolated only twice. However, it is felt that additional work on isolation technique would be beneficial.

Serology: Rising titers to both Bordetella and Pasteurella were demonstrated. The increases were not of great magnitude, seldom greater than a 2- to 4-fold increase.

Discussion: The natural outbreak of "Snuffles" in the rabbit has been observed. The disease has never been consistently produced with the nasal

exudate or the ground suspension of turbinate material. This phase will be the next area to be studied. The question of "single etiology" arises in this study in view of the concomitant rise in titer to both Pasteurella and Bordetella. Attempted isolation of a viral agent in suckling mice, embryonated eggs, weanling hamsters and rabbit kidney has been negative to date. Under closed filtered air systems, the effect of aerosol inhalation of pure cultures of known Bordetella and Pasteurella cultures will be investigated.

Papers Published July 1960 - April 1961:

1. A study of the antigenic relationship between feline and human viruses. R. A. Crandell and J. R. Ganaway. Virol. 11:650-651, 1960.

2. Experimental studies on a new feline virus. R. A. Crandell and S. H. Maden. Am. J. Vet. Res. 21:551-556, 1960.

3. Growth of feline viral rhinotracheitis virus in cultures of feline renal cells. F. F. Ebner and R. A. Crandell. PSEBM 105:153-156, 1960.

4. Susceptibility of primary cultures of feline renal cells to selected viruses. R. A. Crandell, Y. Herman, J. R. Ganaway and W. Niemann. PSEBM 106:542-545, 1961.

5. Experimental feline viral rhinotracheitis in the cat. R. A. Crandell, J. A. Rehkemper, W. H. Niemann, J. R. Ganaway, F. D. Maurer. J. Am. Vet. Med. Assn. 138:191-196, 1961.

Manuscripts Submitted for Publication:

1. Stability of the virus of feline viral rhinotracheitis. G. W. Miller and R. A. Crandell. Amer. J. Vet. Res.

2. Cytochemical studies on the virus of feline viral rhinotracheitis. R. A. Crandell and D. F. Hersey. J. Histo Cytochem.

3. Immunological relationship of selected feline viruses. D. F. Hersey, R. A. Crandell and F. D. Maurer. PSEBM.

Manuscripts in Preparation:

1. Susceptibility of hamster kidney tissue culture to feline viruses.

D. F. Hersey and F. D. Maurer.

2. Biophysical characterization of feline viral rhinotracheitis virus.

R. A. Crandell and A. J. Tousimis.

# ANNUAL PROGRESS REPORT

## Title Page

Project No. 6x61-03-001 - Communicable Diseases.

Task No. 7 --. Preparation and Use of Specific Fluorescent Antibodies.

Name and Address of Reporting Installation:

Armed Forces Institute of Pathology,
Washington 25, D. C.

Name of Division and Branch:

Geographic Pathology Division,
Bacteriology and Immunology Branch.

Professional Authors of the Report:

Principal Investigator:

Joseph F. Metzger, Major, MC, USA

Assistants:

Joe M. Blumberg, Colonel, MC, USA

Chauncey W. Smith, Major, USAF, MSC

Walter F. Malizia, Major, MSC, USA

M. David Hoggan, Captain, MSC, USA

Miss Alice Kase

Reports Control Symbol: MEDDH-288

Security Classification:

Unclassified.

## ABSTRACT

Project No. 6x61-03-001 Title: Communicable Diseases.

Task No.7 --- Title: Preparation and Use of Specific Fluorescent Antibodies.

Name and Address of Reporting Installation:

Armed Forces Institute of Pathology
Washington 25, D. C.

Period Covered by the Report: 1 July 1960 - 30 June 1961.

Authors: Joseph F. Metzger, Major, MC, USA
Joe M. Blumberg, Colonel, MC, USA
Chauncey W. Smith, Major, USAF, MSC
Walter F. Malizia, Major, MSC, USA
M. David Hoggan, Captain, MSC, USA
Miss Alice Kase

Reports Control Symbol: MEDDH-288

Security Classification: Unclassified.

Investigations have been made by immunofluorescent and immune electron microscopy techniques in the detection and pathogenesis of disease processes. Preliminary results indicate a minimum of $10^4$ logs of virus of equine abortion virus for its specific identification by immunofluorescence. The virus has been detected by immune electron microscopy in the nuclei of infected hamster livers. Immunofluorescent techniques are discussed. Thai 16 virus, associated with

hemorrhagic fever, can be detected as well as several mycotic agents that have been implicated with infectious disease processes. Model systems have been used to study the involvement of the animal host with a virus that produces a fatal hepatitis in hamsters. Viral inclusion bodies have been studied by conventional staining, immunofluorescent, and immune electron microscopy techniques. The results are discussed.

BODY OF REPORT

Project No. 6x61-03-001 Title: Communicable Diseases.

Task No. --- Title: Preparation and Use of Specific Fluorescent Antibodies.

Description:

With the advent of more efficient methods of treatment of infectious diseases and the requirements of certain public health aspects of military medicine, it has become apparent that it is highly desirable to investigate new methods for the rapid detection of bacterial, viral and mycotic agents. With the introduction of immunofluorescent techniques their use in rapid diagnosis has been investigated. Also, as an extension of immunofluorescence, the use of immune electron microscopy is being investigated to detect antigen-antibody reactions at the subcellular level by conjugating ferritin, which has a definite micellar structure under the electron microscope, to the antibody. Both techniques are also being utilized to study the associated pathogenesis of certain infectious agents as well.

Progress:

Rapid Identification of Pathogens

Equine Abortion Virus: Equine abortion virus (EAV) produces a rapidly fatal hepatitis in hamsters (16 - 20 hours) which involves more than 99% of the parenchymal cells that show characteristic intranuclear inclusions.

Infected tissues and body fluids from infected animals yield high infectious titres ($10^8$ - $10^{10}$ $LD_{50}$/gm in liver and $10^{10}$ $LD_{50}$ in blood) as well as high levels of CF antigen (1600 to 1900 50% units/gm). These characteristics make it an ideal model system in which to study a virus hepatitis in animals as well as a model to study methods of rapid diagnosis of viral diseases which produce high viremia. In this study to date immunofluorescent and histochemical studies (acridine orange and Fuelgen reactions for nucleic acids) have been used to study the sequential pathological changes occurring in the liver of infected animals. These changes were correlated with the morphological changes as observed with hematoxylin and eosin stain, as well as infectious virus and complement fixing antigen formation in the liver and its subsequent release into the blood stream. In order to more accurately measure infectivity with limited numbers of animals a technique using the mean survival time was developed. Although no changes were noted in sections stained with hematoxylin and eosin and acridine orange or Fuelgen reagent until about 5 or 6 hours after infection, specific antigen was detected by immunofluorescence immediately after injection (15" to 30") and for about 2 hours in cells adjacent to the sinusoids. The antigen then disappeared by 4 hours and reappeared at 5 - 6 hours. It first reappeared in a few nuclear and paranuclear regions. By 8 - 9 hours large deposits

of antigen were noted in the cytoplasm of many parenchymal cells and by 11 hours the whole section appeared brightly fluorescent. The fluorescence then diminished somewhat by the time most animals died (18 - 19 hours). The fluorescence correlated well with the formation of new virus in the liver and the formation of CF antigen. Work is in progress to correlate these changes with the ultramicroscopic pathology and virus particle formation as seen in the electron microscope (immune electron microscopy).

Herpes simplex virus. Studies on this virus have continued on a limited scale. We have been able to adapt this virus to hamsters, causing a general infection marked by a hepatitis which is fatal in 72 - 96 hours. Preliminary studies with ferritin conjugates have demonstrated that we can use immune electron microscopy to localize the antigenic site of the herpes simplex virus particle.

Vaccinia virus. Our attempts to specifically identify this virus both in experimental granulomas and human cases have not been successful because of the great amount of non-specific fluorescence encountered with such tissues. We hope that studies with ferritin conjugates will be of more diagnostic value because this procedure in some measure overcomes many of these inherent difficulties.

Thai 16 virus. In cooperation with the Department of Virology, Walter Reed Army Institute of Research,

immunofluorescent studies are being made of Thai 16 virus. This virus has been isolated from cases of hemorrhagic fever. There is indication that it is one of several viruses related to the disease. In infected primary hamster kidney cell lines the viral infection can be demonstrated with a specific immune serum conjugation to fluorescein isothiocyanate. Preliminary screening indicates an immune cross reaction with Chickengunya virus. There is no cross with eastern equine encephalitis virus.

Mycology. The diagnosis of diseases due to mycologic agents has been unsatisfactory. It is often difficult to isolate the etiologic agent and its demonstration in autopsy material is more than often controversial by the use of the usual conventional staining techniques. The diagnosis of cryptococcosis by immunofluorescent techniques has previously been reported. Improvement of results has been made by producing an antiserum from a small capsule variant which was selected in a defined medium. This immune serum is of a higher degree of specificity and titer which will detect cases of cryptococcosis not detected by previous methods.

There is a definite problem in the evaluation of granulomas in mycologic infections. Morphology and specific staining with conventional methods do not tend to differentiate species. Preliminary investigations have been initiated to evaluate immunofluorescence in the diagnosis of mycologic

agents. An immune serum for a Blastomyces species has been made which seems in preliminary screening to be specific. An immune serum from the spherules of Coccidioides immitis has been produced. Although this serum cross reacts with Cryptococcus capsular material, this may lead to a better understanding of the immunological relationships involved in the diagnosis of mycologic infections. Immune sera have been produced and are being evaluated for Sporotrichum schenckii and Hormodendron species. The schenckii antiserum seems satisfactory for detection of the agent in experimental tissues. Evaluations are in progress for the use of Hormodendrum in the species identification of chromoblastomycosis.

The Candida species have become of greater importance, especially since the advent of antibiotic therapy. The antigenic factors of Candida have been investigated to possibly provide a means of specific identification by immune techniques. By conventional methods it is not feasible to differentiate medically important species from common contaminants. Antigenic analysis of previously studied species as well as ten unstudied species has been completed. Two new antigenic factors have been detected. One of these factors is related to the medically important C. albicans. It is believed that the antigenic structure of the Candida species will lend itself to a specific identification by immunofluorescence of the species of medical importance.

Miscellaneous. Of specific importance in military medicine are other types of agents. Immune sera have been produced for Leishmania braziliensis and L. enriettii. Preliminary evaluations with immunofluorescent techniques indicate a cross reaction between the two species. This evaluation has been made only in the leptomonas stage. Further studies are being made on the detection and differentiation in infected tissues.

Immune electron microscopy. Studies have continued on the detection of antigen-antibody reactions at the subcellular level using this technique. Although the specificity of the ferritin conjugates can be ascertained by immunofluorescent techniques, there has been no simplified method to determine the degree of conjugation of the ferritin label to the immune serum. Also it would be desirable to add more ferritin molecules to the antibody so that the immune reactions could be more easily observed. Using paper electrophoresis (Spinco Model R) it was found that the ferritin-apoferritin complex gave a peak in the $A_2$ globulin position when compared with normal serum. Further experiments have shown that this method presents a reliable and reproducible method of confirming the labelling of an immune serum with ferritin. Unattached ferritin presents a specific peak, while a comparison of an unconjugated with a conjugated serum will demonstrate the attachment of ferritin by a linear increase in the albumin and gamma globulin peak. Antibody can be labelled with a

variable amount of ferritin as indicated by a quantitative relationship in the protein peaks. A modification is now being used wherein twice the amount of ferritin is being attached to the antibody.

Prior to the development of immunofluorescent techniques, viral inclusions were evaluated by their histologic and histochemical patterns. Controversy as to the nature of these inclusions has existed.

Immunofluorescence has made possible the differentiation of cell products from virus and viral antigens, but not the differentiation of virus from viral antigens. Using immune electron microscopy and infectious canine hepatitis virus (ICH) as a model system, the various factors present in viral inclusions were studied. This virus was used as it not only forms inclusions but can be studied with all the techniques commonly used, including immunofluorescence. A continuous line of canine kidney cells (MDCK) was used to propagate the virus on cover slips in Leighton tubes and 32-oz. bottles. A hyperimmune serum was produced in rabbits and conjugated to fluorescein isothiocyanate with the addition of albumin, fraction II, conjugated to Lissamine rhodamine RB 200 as counterstain; also to ferritin for use in immune electron microscopy. Immunofluorescent studies revealed an immune reaction in the nuclear membrane region, similar to the findings of others. There was insufficient resolution to

discern whether the material was composed of virus particles, antigenic masses, or reaction cell products; however, as this is a specific immune reaction, indications are that the inclusions of this virus are composed of virus and viral products. The studies with immune electron microscopy presented a different picture, however, due to the high resolution of electron microscopy plus the advantage of detecting an immune reaction. In the infected cells on which control conjugate had been applied minimal viral particles are observed in the nuclear region, along with some chromatin clumping. In the infected cells, treated with the immune ferritin conjugate, an antigen-antibody reaction can be observed, as indicated by the deposition of the ferritin in the nuclear membrane region. The picture fits very well that observed by immunofluorescence of a typical infection for this virus. As only a minimal amount of virus was observed in the diffuse immune response received, it can be hypothesized that the inclusion bodies of this virus are composed mainly of antigenic material. Immune electron microscopy appears to be a valuable tool for studies of this nature and should provide a means of resolving the problems in the study of the formation of inclusion bodies.

Investigations have been made to detect viruses in infected tissues by immune electron microscopy. Some workers have indicated that the antibody does not penetrate the

nuclear membrane. In our experiments with immunofluorescence we have felt that this theory is not true. Livers of moribund golden hamsters, infected with equine abortion virus, were excised and 1 mm pieces fixed in formalin. Immune serum to the virus that had been conjugated to ferritin was applied. Ultrathin sections were made of methacrylate-embedded material. Nests of virus could be observed in the nuclear material. Ferritin molecules could be observed in the membranes of the virus, indicating a specific immune reaction. The virus could not only be observed in the nucleus but could be specifically identified by the immune reaction. Also, the antibody not only penetrated the nuclear membrane but the antibody molecule was also increased in size by the attachment of the ferritin molecules, showing that molecules of a larger molecular structure can penetrate the nuclear membrane. Further investigations will be made to determine the feasibility of detecting virus in formalin-fixed tissue for the diagnosis of infectious diseases caused by several agents.

Preliminary studies have been made to use this technique as a rapid identification of virus by isolation and contraction with differential centrifugation. Our aim is to provide a specific viral diagnosis in hours instead of days as now required.

Studies on preparation of immune serum. No standard methodology can be used to produce an immune serum for immunofluorescence or immune electron microscopic techniques. The sensitivity of both techniques is such that minor antigenic factors, not detectable by usual serological procedures, now present problems. Our investigations indicate that immunization schedules should be of short duration with increased dosage levels. Although in some instances titers can be of significance, it has been observed that with prolonged immunization minor antigenic factors are enhanced, which give rise to cross reactions. Immune serum has been produced which elicited no titer by conventional methods, but provided a reagent for specific immunofluorescence. Before any immune serum is used as a diagnostic reagent its characteristics must be typified against a panel of any agent that might be encountered in a given specimen. Recent isolates can contain antigenic factors other than the stock cultures used in the basic evaluation of specific diagnostic procedures. To date most evaluations have been made on stock cultures many times removed from an animal host.

The preparation of anti-species globulins, used in the indirect technique in immunofluorescence, has been thoroughly investigated. The technique of Proom (J. Path. & Bact. 55: 419-426, 1943) has been duplicated and found to be the method of choice. In the preparation of over fifteen anti-species

globulins it has been found that his concepts are especially applicable to immunofluorescence. More than two injections can cause undesirable cross reactions. One animal should be used for the preparation of only one lot of serum and discarded.

PPLO and L-forms. It was previously reported that fluorescein-conjugated antisera prepared against tissue culture PPLO strain HEp-2 reacted with 10 tissue culture PPLO strains when tested in infected primary cells and with colonial impressions, but did not react with two human urethral PPLO strains, one saprophytic PPLO strain, one murine PPLO strain, 5 viral agents and 24 bacterial species.

The specificity of the antigen-antibody reaction was demonstrated by adsorption and inhibition studies. Fluorescence was completely eliminated by homologous adsorption but not with heterologous adsorption. The one-step inhibition test also blocked specific fluorescence.

The data indicate that immunofluorescent procedures are specific for PPLO and/or L-form detection. A survey was conducted comparing the detection of PPLO in cell lines by immunofluorescence with detection by cultural procedures. A total of 102 cell cultures from 17 separate laboratories were examined. Of the 102, 92 were continuous cell cultures representing 31 different cell types from 8 animal species and 10 were primary cells representing 7 different cell types from 6 animal species.

PPLO was detected by immunofluorescence in 49 (48%) of the cell cultures examined and from 48 (47%) by cultural methods. There was a 99% correlation between the two techniques. None of the 10 primary cell cultures were positive for PPLO. However, 48/92 (52%) continuous cell cultures were positive for PPLO. Cell cultures propagated in media containing antibiotics showed 46/64 (72%) contamination with PPLO, whereas 2/28 (7%) were found contaminated with antibiotic-free media. Cells grown in media containing sera had 48/86 (56%) contamination while none of 6 cultures propagated in sera-free media had PPLO. It appears from the survey that the incidence of PPLO contamination in cell cultures was lowest when 1) primary cells were used, and 2) when antibiotics and sera were not incorporated in the tissue culture media.

The above data seem to indicate that tissue culture PPLO strains have similar antigenic structure and that they differ from,at least, some human urethral, murine, and saprophytic strains. These findings do not support the hypothesis that PPLO in contaminated cell cultures have a human origin. If the organisms are in fact L-forms of bacteria, then there is reason to suggest that a common bacterium is the source of contamination or that L-forms of at least some heterologous bacteria have common antigens. Immunofluorescence is recommended as a means of detecting PPLO contamination in tissue cell cultures.

Summary and Conclusions:

In the coming year research will continue in the fields of immunofluorescence and immune electron microscopy directed toward the study of the pathogenesis of infectious disease processes. With the high degree of sensitivity of these procedures it becomes apparent that immune sera of a higher degree of specificity must be produced. The ultimate would be a degree of specificity to the individual cellular components. Investigations will be directed toward the alteration of the antigen and/or separation of the specific components by biophysical means to achieve this result. Investigations will continue with the equine abortion virus to study the pathogenesis of hepatitis in the hamster. This study will also be directed toward the identification of specific virus in infected tissue. Further investigation will be made with immune electron microscopy to provide a rapid method of specific identification of purified virus isolated by differential centrifugation. Immune sera have been produced for several viral systems which are to be used.

An attempt to more closely correlate immunofluorescence and immune electron microscopy will be made through interpretation of sites of pathogenesis by ultra-microscopy. This will be attempted with thick sections (0.5 micron) cut on a Porter-Blum microtome. By staining with specific histochemical stains and observing under phase contrast it is felt that architectural comparison can be achieved.

The use of immunofluorescence as a rapid tool for diagnosis will continue as a prime area of investigation. Special emphasis will be placed on rapid diagnosis of viral diseases in tissue culture. Also there will be a slant toward mycotic diseases to provide a means of differentiation of the agents in tissue. This could lend assistance in differentiation of the granulomatous lesions often connected with mycotic infection.

**List of Publications:**


Metzger, J. F., and Smith, C. W., 1960. Rapid identification of Neisseria meningitidis by fluorescent antibody technic. U. S. Armed Forces Med. Jour. 11: 1185-1190.

DeGroot, C. J., Metzger, J. F., Smith, C. W., and Hoggan, M. D., 1960. Demonstration of yellow fever virus in human cell culture by immunofluorescence. Virology 12: 317-320.

Smith, C. W., Metzger, J. F., Zacks, S. I., and Kase, A., 1960. Immune electron microscopy. Proc. Soc. Exper. Biol. & Med. 104: 336-338.

Smith, C. W., and Metzger, J. F., 1961. Studies of ferritin conjugates used in immune electron microscopy. Biochim. Biophys. Acta 47: 587-588.

Hoggan, M. D., Metzger, J. F., and Smith, C. W., 1961. Immunofluorescent and histochemical studies of equine abortion virus hepatitis in hamsters. Fed. Proc., p. 426.

Smith, C. W., Metzger, J. F., and Hoggan, M. D., 1961. Demonstration of antigens in bacterial, viral and mycotic agents by immune electron microscopy. Bact. Proc., p. 123.

Metzger, J. F., Banks, S. I., Smith, C. W., and Hoggan, M. D., 1961. Demonstration of Venezuelan equine encephalitis in tissue culture by immunofluorescence. Proc. Soc. Exper. Biol. & Med. 106 (1): 212-214.

Metzger, J. F., Smith, C. W., and Hoggan, M. D. Demonstration of viral antigens by immune electron microscopy. Pathologia et Microbiologia. In press.

Smith, C. W., and Metzger, J. F. Pitfalls in immune electron microscopy. Experientia. In press.

Blumberg, J. M., Metzger, J. F., Smith, C. W., Hoggan, M. D., and Zacks, S. I. Demonstration of antigenic sites using ferritin conjugates. Exhibit, Meeting of Amer. Soc. Clin. Path., Chicago, Sept. 1960, and Meeting of Amer. Soc. Microbiol., Chicago, April, 1961.

# ANNUAL PROGRESS REPORT

PROJECT TITLE: 6X59-01-001 Traumatic Surgery and Shock

TASK: 8 Structure and Function of Ocular Tissue

REPORTING INSTALLATION: Armed Forces Institute of Pathology
Washington 25, D. C.

DEPARTMENT AND DIVISION: Department of Pathology
Division B-General and Special Pathology
Ophthalmic Pathology Branch

PERIOD COVERED: 1 September 1960 - 30 June 1961

AUTHORS:

PRINCIPAL INVESTIGATORS: Benjamin Rones, M.D. and
Lorenz E. Zimmerman, M.D.

ASSISTANT: Ben S. Fine, M.D.

REPORTS CONTROL SYMBOL: (RCS-MEDDH-288)

SECURITY CLASSIFICATION: Unclassified

## ABSTRACT

**PROJECT TITLE:** 6X59-01-001 Traumatic Surgery and Shock

**TASK:** 8 Structure and Function of Ocular Tissue

**REPORTING INSTALLATION:** Armed Forces Institute of Pathology

Washington 25, D. C.

**PERIOD COVERED:** 1 September 1960 - 30 June 1961

AUTHORS: Benjamin Rones, M.D. and Lorenz E. Zimmerman, M.D.
ASSISTANT: Ben S. Fine, M.D.

REPORTS CONTROL SYMBOL: (RCS MEDDH-288)

This progress report covers a period of less than 10 months since the present research was activated only on 1 September 1960. Much of this time was of necessity spent in organizing the laboratory (recruiting personnel, purchasing equipment, modifying existing facilities, etc.) and this job has not yet been completed. Considerable progress has been made nevertheless in our electron microscopic studies of ocular tissues, particularly the ciliary body, vitreous, retina, and retinal pigment epithelium. Magnificent preparations of the normal human retina have been obtained and two reports of observations based on this work are being completed for publication.

The first of these papers makes a comparison of the ultrastructure of the "limiting membranes" of the retina, while the second correlates histochemical and electron microscopic observations of the interstitial mucopolysaccharide of the rod and cone layer.

6X59-01-001 Traumatic Surgery and Shock: Structure and Function of Ocular Tissue

## DESCRIPTION

Our understanding of the pathogenesis of many important ophthalmic conditions (eg. cataracts, glaucoma, degenerations of the vitreous and retina, retinal detachment, idiopathic optic neuritis, etc.) has been seriously impaired by our lack of detailed knowledge of structure and function of normal ocular tissues. Included in this lack of information is our ignorance of important species variations in form and function. The aims of this research are: (1) to provide more detailed information concerning the ultrastructure of all ocular tissues in man and in certain important experimental animals which will be required for future research; (2) to correlate ultrastructure of ocular tissue with their function; and (3) to investigate changes in normal form and function in relation to pathogenesis of certain lesions of military importance (eg. environmental and occupational trauma -- severe heat, cold, malnutrition, electromagnetic radiations, etc.).

## PROGRESS

During the first several months after this grant was received, equipment was ordered, the laboratory organized, and technical assistance recruited. The latter proved to be a major obstacle to rapid progress since the extremely limited funds available were totally inadequate to attract experienced personnel. An attempt is being made to train unskilled laboratory assistance and it is still too early to evaluate the results. Shortly after the job of organizing the laboratory was begun, one of our

key research associates, Mr. Anastasios J. Tousimis, Biophysicist (GS-11), announced his resignation to return to school to complete his Ph.D. work. His resignation was effective 31 January 1961. This has required some reorientation of the research with a temporary de-emphasis on some of the biophysical aspects (not including electron microscopy).

Work accomplished during this period can be summarized under two headings:

1. Continuation of electron microscopic studies already started. These studies were concerned mainly with the ciliary body, anterior vitreous, and suspensory ligaments of the lens. A comparison was made of the cytologic characteristics of the pigmented and nonpigmented ciliary epithelium passing posteriorly from the iris root, through the pars plicata, and into the pars plana. While certain cytologic differences have been observed (eg. at the tips of the ciliary processes and in the valleys between processes), the significance of these observations remains to be determined. The attachments of the anterior vitreous and suspensory ligaments of the lens to the ciliary epithelium and lens capsule have been studied. These structures appear to be inserted into the basement membranes of the nonpigmented ciliary epithelium laterally and into the lens capsule centrally. In these more recent studies the use of new improved methods of tissue preparation have made possible better electron micrographs of these tissues than have been previously published. The reproducibility of results and greater reliability of data obtained, permit more valid conclusions.

2. Initiation of new studies. These have been concerned mainly with an investigation of the fine structure of the human retina including the vitreous attachments on its inner surface and the pigment epithelium with its relationships to the visual cells and to the choroid externally. Current thought suggests that the pigment epithelium plays a major role in the transport of metabolites between the vasculature in the choroid and the visual cells in the retina. Because of its pigment granules, this tissue also plays a major role in the pathogenesis of certain injuries (eg. atomic flash and eclipse burns of retina). Experimentally the human retina, as well as the animal retina, can be "photocoagulated" using Meyer-Schwickerath's instrument (available at Walter Reed). One human retina (eye enucleated because of malignant melanoma) given subclinical dosages of photocoagulation has already been prepared for electron microscopy.

Magnificent preparations (electron micrographs and histochemical reactions) of the normal human retina have been obtained and two reports of observations based on this work are being completed for publication.

The first of these papers makes a comparison of the "limiting membranes" of the sensory retina and retinal pigment epithelium. The internal limiting membrane of the retina and the cuticular portion of Bruch's membrane are considered analagous structures. The former is the basement membrane elaborated by the inner (basal) portion of Muller's cells (specialized retinal astrocytes) while the latter is the basement membrane of the retinal pigment epithelium. In contrast with these true basement membranes, the "outer limiting membrane" of the retina and

the fenestrated membrane of the pigment epithelium are not true membranes but rather a series of terminal bars. In the case of the outer limiting membrane, the terminal bars are adhesion plates between the visual cells and the outer ends of adjacent Muller's cells. In the case of the fenestrated membrane of the pigment epithelium the terminal bars connect adjacent cells of the pigment epithelium.

The second paper based in part on these studies correlates the new observations made by electron microscopy of the retina with those obtained by histochemical studies of the retinal mucopolysaccharides. The visual cells are imbedded in an intercellular matrix which stains deep blue with such methods for acid mucopolysaccharides as Hale's colloidal iron reaction, alcian blue (pH 2.6), and Astrablau (pH 0.4-2.8). The staining reaction is not affected by pretreatment with hyaluronidase or diastase. Use of the Abul-Haj reaction indicates the presence of sulfated acid mucopolysaccharides. Electron microscopy confirmed our assumption based on light microscopy that the mucoid material is interstitial. Between the outer limiting membrane of the retina and the pigment epithelium, the visual cells are separated by rather large amounts of interstitial mucoid material. Insite the external limiting membrane, however, the retinal cells are in extremely close juxtaposition and no interstitial ground substance is observed - either in electron micrographs or by histochemical methods.

## SUMMARY AND CONCLUSIONS

1. Progress in our understanding of the pathologic anatomy, pathologic physiology, and pathogenesis of such important ocular diseases as cataract formation in the lens, detachment of the retina, glaucoma, and optic neuritis must be dependent upon the acquisition of more basic

information concerning the fine structure of ocular tissues and a better correlation of ultrastructure with function, first in the normal, then in the pathologic eye.

2. The necessary laboratory facilities and personnel are being organized for this work and tangible progress has already been made.

3. Two papers are being completed and will soon be submitted for publication.

## PUBLICATIONS

1. Fine, B. S.: Limiting membranes of the retina and pigment epithelium: An electron microscopic study, in preparation for Archives Ophthalmology.

2. Zimmerman, L. E., and Fine, B. S.: Histochemical and electron microscopic studies of the ground substance of the retinal rod and cone layer, in preparation for LABORATORY INVESTIGATION (AFIP Centennial issue).

## DISTRIBUTION

As per OTSG Admin. Ltr. 705-2